Role of Arbuscular Mycorrhizal Fungi in Fighting Soil Salinity

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Declaration

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree. This thesis is a result of my own investigations, except where otherwise stated. Other sources are acknowledged with explicit references. A bibliography is appended.

Signed ……. (Candidate)

Date …….

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Abstract

The evaluation of mycorrhizas role in fighting soil salinity and sustaining plant growth under stress was extensively studied. However, very few studies are currently available to address the role of different mycorrhizal species or the consequences of using single or mix consortia of mycorrhizas in fighting soil salinity. To the best of my knowledge, no studies are available that examine the combined effect of using mycorrhizas under salt stress on the plant second generation. In the present study, the first set of experiments was conducted using mixed species of mycorrhizas provided by a specialised commercial company. The second set of experiments utilised individual species of *Glomus etunicatum* and *G. mosseae*. Two salt types were used to examine the effect of salt chemical structure on mycorrhizal-plant interactions. NaCl salt was used initially followed by a mixed combination of salts in subsequent experiments. Three levels of salinity stress were applied which were categorised into low (1-4 dS/m), medium (5-8 dS/m) and high salinity effect (> 9 dS/m). The focal plant used in this study was Ribwort Plantain (*Plantago lanceolata*). All experiments were conducted in controlled condition (controlled room & glasshouse) as well as in field conditions during the summer season. Different vegetation parameters were recorded for the first generation plants at the end of each experiment and seed germination testing was conducted for the second generation. The mix of commercial mycorrhizas failed to colonize the plant under the controlled room condition; however, in the glasshouse and the field areas the mixed mycorrhizas successfully colonized the plant roots. Root colonization was also successfully observed with the individual species of mycorrhizas under the salt stress in both controlled and field condition. Using mixed salt reduced plant growth more severely than using NaCl with the different mycorrhizal species. Different treatments of mycorrhizas were helpful for the plant only up to the medium level of salinity, while at the higher level of stress mycorrhizas failed to support the plant. The effect of mycorrhizas on the plant second generation was weak under different salinity stress, and in some experiments it failed to show any positive results on the second generation growth. Overall, the theory of Functional Complimentarity for mycorrhizal species was not supported in this study as using single species of mycorrhizas were more successful than mixed species under salinity stress in some situations.

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Chapter 1

Introduction

1.1 Salinity stress and plants

The accumulation of salts in the soil is one of the major threats facing crop production worldwide (Nasim 2010). It has been estimated that 7% of the earth's land is exposed to high soil salinity levels (Lazano *et al.*, 1996). Salinity refers to the content of water-soluble salts, primarily sodium, potassium, magnesium, calcium and chloride, in the soil. ECe (the electrical conductivity of a saturated paste extract) values of 4 dS/m or more, which is equivalent to 40 mM NaCl, are characteristic of saline soils (USDA – ARS 2008). According to some estimates, 77 million hectares of irrigated land have been severely affected by salinity, markedly reducing the agricultural potential of 5% of the world's land (Munns *et al.*, 1999). Semi-arid and arid areas around the globe are most affected by soil salinity, with consequent reductions in crop productivity (Giri *et al.*, 2003; Al-karaki 2006). It is expected that with global warming the salinity problem will spread further to affect half of the world's cultivated areas (Gamalero *et al.*, 2009). It is not only plants that are affected by salinity; populations of soil microorganisms can be damaged and soil properties can also be severely compromised (Zhu 2002; Yuang *et al.*, 2007). Salt-affected soil is characterized by an excessive accumulation of salt ions, namely Na^{+2}, Na^{+}/K^{+}, Mg^{+2}/Ca^{+2} and Cl^{-}/NO^{-3} (Meybodi & Ghareyazi 2002). Several factors underlie this problem, including irrigation using groundwater with a high salt content, the addition of fertilizers and high evaporation rates due to rising temperatures (Juniper & Abbott 1993; Cantrell & Linderman 2001; Mouk & Ishii 2006).

Accumulating salt beyond certain critical levels can cause many physiological and biological problems for plants (Taiz & Zeiger 2006). For example, salt stress can cause changes in the photosynthetic rate (Lovelock & Ball 2002). Plants normally are able to produce more carbon by increasing their rate of photosynthesis. However, under salt stress conditions plants decrease the opening of their stomata in order to conserve water, which in turn alters their rate of photosynthesis and reduces the plants' carbon fixation. Another method by which plants overcome salinity is by lowering cell turgor to conserve water (Rozema 1991). One example is the highly salt tolerant *Salicornia*, which has a great ability to retain water and prevent dehydration (Balnokin *et al.*, 2005).

Nonetheless, salts can still disturb apoplastic transport between the cells due to ion accumulation and prevent water movement (James *et al.*, 2006). With respect to plant tissues, elevated salinity makes the tissues more rigid and changes the cell wall elasticity (Touchette 2006). Additionally, salinization of the soil with the concomitant increase in plants' Na^+ and Cl^- levels is associated with multiple internal problems due to inhibition of protein and enzyme synthesis (Fricke *et al.*, 2004). Furthermore, salinity damages the soil particles, reducing porosity and the ability of plants to absorb water (Rengasmay 2002). Salinity also decreases photosynthesis by changing leaf pigmentation, which in turn leads to diminished plant biomass (Yeo *et al*., 1991). Different experiments addressing the effects of salinity have shown that it attenuates CO_2 exchange in the leaves (Bongi & Loreto 1989; Centritto *et al.*, 2003; Loreto *et al*., 2003). Salt has also been shown to disturb plant cells and reduce their turgor, thereby shortening their life span (Serrano 1999). Increased salt accumulation within plants results in a state of water deficit, which disrupts cell osmosis and leads to loss of turgor. On the other hand, salinity can affect plant growth through the production of ethylene (Shibli *et al.,* 2007) and can also lower the quality of fruit production and plant yields (Cerda *et al.,* 1990; Dasberg *et al.,* 1991). Leaf area and leaf diameter can be significantly reduced under salinity stress as well (Yeo *et al.,* 1991; Sumer *et al.,* 2004). Furthermore, salinity may affect plants indirectly by increasing the alkalinity of the soil, making it harder for plants to absorb nutrients (Pankhurst *et al.,* 2000).

According to Munns & Tester (2008), there are three distinct mechanisms that enable plants to tolerate salinity stress. The first mechanism involves enhancing the tolerance of the plant to osmotic stress under salinity, thus potentiating leaf growth and stomatal conductance. The second mechanism is based on Na^+ exclusion, which in turn restricts it from accumulating to toxic levels within the leaves. Tissue tolerance to accumulated Na^+ is the third mechanism that helps plants overcome salt stress. This process entails the sequestration and accumulation of the ions in the cytoplasm so that salt particles will not interfere with physiological and biochemical processes during plant development (Munns & Tester 2008). However, with the exception of halophytes (plants that survive highly saline habitats; reviewed in Flower & Colmer 2008), the majority of plants and crops still fail to resist soil salinity concentrations above 4 dS/m (USDA – ARS 2008).

To address the above-mentioned impacts on plant growth and agricultural losses, novel methods for overcoming salinity are being investigated and introduced. There has

been a growing interest in identifying new plants that can cope with salinity, and at the same time be suitable as new crop plants for human and animal consumption (Gallagher 1985; Glenn & O' Leary 1985). Another approach is to breed existing crop plants specifically to develop strains that can withstand salt stress (Guartero & Fernandez-Munoz 1999). Recently, genetic engineering has also taken on an important role in overcoming salinity by designing plants with genes that enable adaptation to high salinity conditions (Sanan-Mishra *et al.,* 2005; Wu *et al.,* 2005; Wei-Feng *et al.,* 2008). Apart from these biological methods to combat soil salinity, mechanical methods may also be used. These include using chemicals to leach excessive salts from soil, and the use of desalination machines to remove salts from irrigation water (Muralev *et al.,* 1997). However, the problem remains that conventional methods for fighting soil salinity are expensive, and most farmers in developing countries cannot afford the financial burden (Cantrell & Linderman 2001).

1.2 Biology of arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are amongst the most common soil fungi and the majority of plant species have associations with AM fungal species (Selvaraj & Chellappan 2006). It is thought that about 80% of vascular plants form AM associations (Hodge 2000). Mycorrhizal fungi can be found in all ecosystems (Read 1991) and have been found in arid areas, tropical regions, sub-polar habitats and even in aquatic ecosystems (Nielsen *et al.,* 2004). Plant–mycorrhizal associations are extremely ancient and can be traced back over 400 million years (Remy *et al*., 1994). Each plant species has a different degree of dependency on mycorrhizas; as an example, faba bean (*Vicia faba L.*) is highly mycorrhizal and depends on its fungal association for growth and establishment (Talaat & Abdallah 2008). Mycorrhizal fungi can help plants to survive and grow under different environmental conditions, and also help plants increase their reproductive output (Bolandnazar *et al.,* 2007). The basic elements of the symbiosis are that the plant provides the mycorrhiza with carbohydrates while the fungi supplement the plant with certain nutrients needed for growth (Selvaraj & Chellappan 2006). It has been estimated that in compensation for the additional nutrients and water provided by mycorrhizas, a plant must provide 20% of its fixed carbon to the roots for mycorrhizal establishment and maintenance of the association (Tunc – Ozdemir *et al*., 2009).

AM species are very important for plant biodiversity and the health of ecosystems (Van der Heijden *et al.*, 1998a), and can help plants withstand different forms of environmental stress. It has been reported that AM fungi enabled plants to grow and reproduce in heavy metal contaminated sites (Malcova *et al.*, 2003; Rydlova & Vosatka 2003; Sudova *et al.*, 2007) as well as increasing the uptake of soil moisture by the host plant, thereby allowing them to better withstand the effects of drought (Reid & Bowen 1997; Auge 2001). Moreover, mycorrhizas can help plants to resist extreme temperatures (Zak *et al.*, 1998) as well as reduce the stress of herbivore attacks (Gange & West 1994; Gehring & Whitham 2002; Wamberg *et al.*, 2003) and also protect plants from many diseases and pathogens (Sharma *et al.*, 1992; Newsham *et al.*, 1994). AM fungi were classified recently as belonging to the phylum *Glomeromycota* (Gerdemann & Trappe 1974). Unfortunately, there are no accurate estimates of the species richness of mycorrhizal fungi, but some estimates reach 150 species (Morton & Benny 1990).

AM fungal associations are composed of three main structures. First, hyphae work as external filamentous arms searching for nutrients around the root zone (Hodge 2000). Second, there are specialised vesicles within the root, which are thought to be storage organs, especially for lipids (Hirsch & Kapulnik 1998). Arbuscules are the third important part of the AM association. They are branched intercellular structures, resembling trees, and are the main functional site of phosphorus and other nutrient exchange in the root system (Smith & Read 1997).

1.3 Arbuscular mycorrhizas and plant salinity interaction experiments

Due to the multiple benefits of AM species for individual plants and ecosystems, there has been an attempt to transfer these benefits of AM fungi into commercial areas, especially those dealing with agriculture, plant production and land restoration (Von Alten *et al.*, 2002; Vosatka & Dodds 2002; Gianinazzi & Vosatka 2004). It has been recognized that mycorrhizas can be used to help plants overcome extreme environmental conditions, such as saline environments (Hildebrandt *et al.*, 2001), and a number of AM species have been found living in saline habitats (Wang & Liu 2001). According to some estimates, around 50% of plants living near shorelines possess mycorrhizal associations in their root systems (Cooke & Lefor 1998). Similarly, several species of AM were discovered in salt marsh plants (Sengupta & Chaudhuri 1990;

Hoefnagels *et al*., 1993; Hildebrandt *et al.,* 2001). Even in very saline sites reaching more than 150 dS/m of electrical conductivity, there are species of AM that are able to survive such hostile conditions (Aliasgharzadeh *et al.,* 2001). It was discovered that certain species of AM fungi had greater spore germination with increased salinity (Tresssner & Hayes 1971). Therefore, recent studies have recommended using AM species to combat soil salinity and enhance plant production (Yano-melo *et al*., 2003; Rabie 2005; Jahromi *et al*., 2008). Using AM fungi as biological tools to fight soil salinity has practical benefits and should be financially cost effective.

There are different mechanisms by which AM fungi can help plants cope with salt stress. For example, they can enhance soil nutrient absorption by plants (Asghari *et al.,* 2005). Canterall and Linderman (2001) showed that the addition of AM fungi to lettuce and onion plants resulted in increased accumulation of phosphorus under conditions of salinity stress. Furthermore, AM can affect the ionic balance of plants, especially with regard to Na^+ and Cl^- (Giri *et al*., 2007). The same authors, when studying the effects of salinity on Arabic gum tree (*Acacia nilotica*), showed that with increasing salinity levels, AM fungi reduced Na ions in the plant but increased K ions. This fungal association can also protect plant enzymes from damage under conditions of high salinity (Rabie & Almadini 2005). It was found that the addition of AM fungi increased the activities of both nitrogenase and phosphatase enzymes in bean plants (*Vicia faba*) (Rabie & Almadini 2005). Furthermore, the addition of AM to tomato (*Lycopersicon esculentum*) under conditions of salinity improved anti-oxidant enzyme production, thus protecting cell membranes from damage (He *et al.,* 2007). AM fungi can also improve the secretion of different types of hormones, one of them being abscisic acid (Danneberg *et al.,* 1992). Mycorrhizal effects on hormones are important, as these hormones can enable plants to overcome many environmental stresses (Zhang *et al*., 2006). For example, inoculation of lettuce (*Lactuca sativa*) with *Glomus intraradices* induced enhanced levels of hormones in these plants under conditions of salinity stress and this in turn affected the regulation of stomatal closure (Jahromi *et al*., 2008). Salinity may also induce drought conditions for plants, so AM fungi may also help plants increase water uptake (Ruiz – Lazano & Azcon 1995). The addition of mycorrhizas to leek (*Allium porrum*) increased the surface area of the roots, thereby increasing water absorption by the plants (Berta *et al.,* 1990). The efficiency of water use in lettuce plants improved significantly with the addition of mycorrhizas under salt stress (Ruiz – Lazano *et al.,* 1996). Plant chlorophyll concentration may also be affected

by AM fungi under conditions of salinity stress (Sannazzaro *et al*., 2006). In mycorrhizal zucchini plant (*Cucurbita pepo L.*) leaves, chlorophyll content was considerably higher than that found in control plant leaves when salinity was also present (Colla *et al.,* 2008). Finally, in legumes, AM associations can enhance nodule performance under salt stress as well. It was found that the addition of AM fungi to pigeon pea (*Cajanus cajan*) enhanced root nodulation and led to more nitrogen fixation, enabling plants to overcome salinity stress (Garg & Manchanda 2009). However, there have been other experiments in which the presence of AM fungi did not show any positive effect on plants under salinity stress. Application of mycorrhizas to evaluate the resistance of citrus lemon (*Citrus limon*), sour orange (*C. aurantium*), rough lemon (*C. jambhiri*), red lemon (*C. volkameriana*), alemow (*C. macrophylla*), rangpur lime (*C. limonia*) and sweet lime (*C. limettoides*) to soil salinity did not give any positive results (Levy *et al*., 1983). In another experiment, AM fungi were added to three root stocks of sweet orange (*Citrus sinensis*), citrange (*X citroncirus*) and sour orange (*C. maxima x C. reticulate*) under conditions of high salinity; however, these plants showed no difference in growth compared to the controls (Hartmond *et al*., 1987).

It is suspected that the specific origin of AM fungi and their genetic makeup is important for adapting to, and resistance against, soil salinity. Several studies on the isolation of AM fungi from saline habitats and estuaries have indicated that specific mycorrhizal species are better adapted to saline conditions. One in particular is *Glomus geosporum*, which comprises up to 80% of AM fungal species occurring in such extreme habitats (Hildebrandt *et al*., 2001; Landwehr *et al*., 2002; Carvalho *et al*., 2004). In another study, two strains of *G. mosseae* from saline and non-saline origins were isolated and used to inoculate cotton (*Gossypium arboreum*) roots under saline conditions. The results showed that the mycorrhizal strain originating from the saline habitat was far better adapted to conditions of salinity and conferred a much greater benefit to the host plants (Tian *et al.,* 2004). Meanwhile, experiments on two AM isolates, *G. geosporum* from a saline area and *G. intraradices* from an adjacent site (not suffering from salinity) on *Plantago lanceolata* plants, revealed that *G. geosporum* was much more efficient in enabling plants to cope with salinity stress (Grzybowska 2004). A further study isolated *G. geosporum* from an area of high salinity and found that when this AM strain was used as inoculum, it produced a remarkable improvement in fleabane (*Conyza bilbaoana*) growth and a significant increase in phosphate in plant tissues under salinity-induced stress. This was in comparison to inoculation by a similar

species of AM fungi from a non-saline source (Oliverira *et al*., 2010). However, there are some exceptions, such as the study of Cantrell and Linderman (2001) that found that AM fungi collected from non-saline sources performed better under salinity stress than AM fungi collected from a saline habitat. A separate study showed that an isolate of *G. geosporum* isolated from a salt marsh did not help various plant species cope with salinity stress under experimental greenhouse conditions (Fuzy *et al*., 2008). Investigating the effects of AM fungi from different sources on the resistance of tomato (*Solanum lycopersicum*) to salinity, it was shown that AM fungi from non-saline sources increased shoot biomass even though the Cl concentration in the root increased, while mycorrhizas from a saline origin decreased the concentration of Cl in leaves, although plant biomass was also reduced (Copeman *et al.*, 1996). Thus, even if the AM fungal species is adapted to salinity stress, it may still not provide beneficial effects to host plants in all situations. Juniper and Abbot (2006) suspected that the failure of some AM fungi to grow under saline conditions was due to inhibition of spore germination and hyphal spreading. Moreover, salinity stress reduced root exudate production, thus decreasing the attraction of AM fungi to the root and leading to reductions in fungal colonisation (Gamalero *et al*., 2009).

It is important to realize that in mycorrhizal–plant interactions, each fungal species interacts differently with different plant species. Several studies have confirmed that different plants have specific AM species that positively influence their growth, while other AM species can harm the plant (Streitwolf- Engel *et al*., 1997; Van der Heijden *et al*., 1998). One reason why AM species have differing effects on plants appears to be due to their ability to absorb phosphorus (P) from the soil at different rates and the amount which is transferred to the plant (Jakobsen *et al.*, 1992). It is known that different AM species have varying lengths of hyphae within the rhizosphere, thus leading to differential absorption of P (Jakobsen *et al.*, 1992). As an example, *Plantago* plants showed a far greater positive growth rate when associated with *Acaulospora morrowiae* and *Archaeospora trappei* than associated with *Scutellospora calospora* mycorrhizas (Bever *et al*., 2002). In a salinity stress study using three species of mycorrhizas (*G. mosseae*, *G. intraradices* and *G. fasciculatum*) on bean (*Phaseolus vulgaris*), it was found that *G. mosseae* was the most beneficial for increasing plant growth and nutrient accumulation compared with the other species (Ciftci *et al*., 2010). Meanwhile, a study of elevated salt stress on olive (*Olea europaea*) trees inoculated with different species of AM fungi (*G. mosseae*, *G. intraradices* & *G. claroideum*),

showed again that *G. mosseae* was the most efficient species of AM in helping trees avoid salinity damage (Porras-Soriano *et al.,* 2009). Testing the germination rate of spores of different AM fungal species under conditions of salinity stress showed that *S. calospora* can resist salinity under light and medium stress, but *Acaulospora laevis* failed to germinate even under low salinity stress (Juniper & Abbott 2006). Under field conditions, *G. etunicatum* enabled different varieties of wheat (Roshan variety & Kavir variety) to overcome salinity stress better than *G. mosseae*, whilst the least effective was *G. intraradices* (Daei *et al*., 2009). Interestingly, even in the same experiment, different species of AM fungi can produce different benefits on the same host plant. A salinity experiment on orange (*Citrus tangerine*) with *G. mosseae* and *G. versiforme* showed *G. mosseae* was better able to help plants overcome physiological problems associated with salinity, while *G. versiforme* had a stronger association with the roots under salinity stress (Wu *et al.,* 2010). Similar results were obtained by Ruiz-Lozano and Azcon (2000), where *G. deserticola* improved plant nutrition under salinity stress, but *Glomus sp.* increased root development and growth. Thus, different AM fungal species have variable responses with certain plant species under salinity stress, and there are no general rules that can be applied currently to predict the outcome of experiments studying these fungi and soil salinity.

It is not just the identity of the AM fungal species that is important. Using a mix of AM fungal species can produce different results compared with single inocula. For example, using a mix of *G. fasciculatum* and *G. macrocarpum* under salinity stress conditions enhanced earleaf acacia (*Acacia auriculiformis*) growth more than when using a single species of AM fungus under the same conditions (Giri *et al*., 2003). Another experiment using the salt marsh plants smooth cordgrass (*Spartina alterniflora)* and big cordgrass (*S. cynosuroides*) showed that a commercial product of mixed AM species had positive effects on plant establishment (McHugh & Dighton 2004). The benefits of using a consortium of AM species are seen not just in improved nutrient uptake, but also in root structure (Berta *et al*., 2002). Using a mix of AM species may further benefit the plant, as different mycorrhizal species add their own benefits to the association in what is called functional complementarity (Koide 2000). However, the converse may also be true. There have been some experiments that show that a single AM species can be more beneficial than a mixture of species. A mix of AM species gave lower stomatal conductance for plants under conditions of salinity stress in comparison with the same plants associating with a single species *Gigaspora margarita*

(Cho *et al.*, 2006). It has been reported in several studies that multiple species of AM fungi can have negative effects on plants due to AM fungal species competition (Sen *et al.*, 1990; Pearson *et al.*, 1993). For example, it was shown that colonization competition occurs between the hyphae of *Gi. margarita* and *G. proliferum* (Cano & Bango 2005). In this competition, a species of AM fungi can be rejected in favour of either one species or several others in association within the root (Wilson & Trinick 1983). It was revealed by using three species of AM fungi on leek plants (*Allium porrum*) that *Glomus sp E3* could not compete with two other fungi (*Glomus mosseae* and *G. caledonium*) when trying to colonise roots (Hepper *et al.*, 1988). Meanwhile, another experiment with *Plantago lanceolata* and different species of AM showed that *Scutellospora calospora* was the most vigorous competitor in the root system in contrast to other *Glomus* species (Bennett & Bever 2009).

It has also been found that different types of salt and ions have different effects on the response of AM fungi and their behaviour within plants. A comparison between two salts ($NaNo_3$ and Na_2SO_4) on AM fungal spore germination indicated that germination was affected in different ways (Juniper & Abbott 1993). In a similar experiment, NaCl allowed a higher germination rate of AM spores than did KCl (Hirrel 1981). Mor and Manchanda (1992) studied barley (*Hordeum vulgare*) with a mixture of salt types and found that SO_4 was less harmful than the Cl^- salt ion to barley growth. However, in Europe, the colonization levels of AM fungal species in various salt marshes containing different types of salt were comparable (Wilde *et al.*, 2009). Thus, in salinity studies, it is important to use other types of salts as well as NaCl to achieve more meaningful results.

The level of salinity stress can have varying effects on different species of AM fungi, with some species being most effective at high salinity levels, whilst others function at lower salinity. Jahromi *et al.*, (2008) demonstrated that *G. intraradices* performed well under light and medium salinity stress, but at a higher salinity level reaching 10 dS/m EC the species' activity was disrupted. Using a combination of AM fungal species at a high salinity level, *G. mosseae* was better adapted than *G. claroideum* and *G. intraradices*, but at lower salinity levels the latter two species of AM were better adapted than *G. mosseae* (Peng *et al.*, 2011). The stomatal conductance of lettuce (*Lactuca sativa*) plants was positively influenced by colonization by *G. mosseae* under low salinity stress, but at high salinity *G. fasciculatum* was better at influencing conductance (Ruiz-Lozano *et al.*, 1996). In the same study, *G. deserticola* enhanced

water use efficiency under conditions of low salinity stress, but at high salinity *G. mosseae* was more efficient. Moreover, the transpiration rate in *Lactuca* plants colonised by *G. mosseae* was higher at low salinity levels, but at high salinity the transpiration rate was reduced in the presence of *G. mosseae* and increased with *G. fasciculatum*.

The environmental conditions, or microclimate, where an experiment is carried out are important in AM fungi-salinity interactions. Unfortunately, most studies have been done under controlled conditions such as in greenhouses or controlled environment rooms, and not in realistic situations out in the field. Salinity experiments on different wheat (Roshan, Kavir, and Tabasi) cultivars and AM fungi under field and greenhouse conditions gave similar results (Mardukhi *et al*., 2011). Another study of wheat comparing the effects of indigenous soil AM fungi under both field and greenhouse conditions produced similar results in the case of phosphorus uptake (Covacevich *et al.*, 2007). Pringle and Bever (2008), working in North Carolina under both field and lab conditions, concluded that the effects of AM fungi in controlled conditions can be similar to those recorded in the field. They therefore recommend assuming that data from controlled conditions paralleled those recorded in the field. A study on a Portuguese salt marsh hypothesised that environmental factors do not have an effect on the identity and behaviour of AM fungi during their association, and that the only important aspect in determining the role of mycorrhizas was the identity of the plant species (Carvalho *et al.*, 2001). One study reported an enhancement in AM fungal resistance to salinity in the presence of soil rhizobacteria, suggesting that under field conditions mycorrhizal benefits are greater than in controlled situations (Abdel-Rahman *et al.*, 2011). On the other hand, other experiments have not shown advantages of AM fungi under field conditions compared with controlled conditions in a greenhouse (Fitter 1984). Investigating the role of phosphorus addition to soil under greenhouse conditions, there was a clear decrease in mycorrhizal root colonisation (Mendoza & Pagani 1997; Cornwell *et al.*, 2001). However, in the field, colonisation was unaffected by the amount of phosphorus within the soil (Sanders & Fitter 1992). Some species of AM fungi can change their salinity tolerance in the field when they are subsequently grown in a greenhouse. For example, several AM fungal species were collected from salty areas around California and Nevada for use in studies with tomato (*Solanum lycopersicum*) in a greenhouse. However, only *G. fasciculatum* showed a beneficial effect, while the rest of the AM fungal species failed to enhance plant growth under

controlled conditions (Pond & Menge 1984). Many reasons have been proposed for the different behaviour of AM fungi in the field vs. controlled environments. In the field, any individual plant may be connected with other plants via the mycorrhizal mycelium (Hickman & Mooney 1982). This interconnection between plants may reduce the benefits of the AM fungi because it spreads resources between the plants (Fitter 1984). Mycorrhizal hyphae can also be a source of food for soil microarthropods, reducing the efficacy of the association (Schenk *et al.,* 1975; Warnock *et al*., 1982). Also, variable levels of moisture in the field may affect AM fungi differently compared to regular watering under controlled conditions (Fitter 1984). Under field conditions, seasonal variations in weather can have an impact on AM species and their effects on plants (Karthikeyan & Selvaraj 2009). Finally, there is potential competition between introduced AM fungi and the local field microflora that may lead to negative results when introducing inocula of AM fungi (Bowen & Rovera 1976).

1.4 Aims of the thesis

The overall aim of this thesis was to investigate the effect of using mycorrhizal species to combat soil salinity and enhance plant growth under conditions of salinity stress. I hypothesised that mycorrhizas can help plants overcome salinity stress and enhance their growth. There are three research-focused areas in this thesis. The first research objective was to investigate the interaction between salinity and mycorrhizas on the growth of first and second generation plants. Although the effect of mycorrhizas on first generation plants has been extensively studied, very few studies on the role of AM fungi in enhancing the growth parameters of second generation offspring are currently available (Koide 2010). There are some published studies on the effect of mycorrhizas on plant reproductive organs such as flowers, fruit quality and seeds, but, to the best of my knowledge, none of these studies continued to investigate seed germination and second generation seedling performance. It has been proposed that AM fungi enhance the growth of second generation plants by increasing the amount of phosphate in the seed (Khanizadeh *et al.,* 1995) or by manipulating plant hormones (Miller *et al.,* 1987). Thus, experiments focused on whether AM fungi and salt stress could affect not only the growth of parent plants but also that of their offspring, through seedling performance. The second objective was to examine whether using mixed species of AM

fungi or single species may give different results under conditions of salinity stress. I hypothesised that combinations and single species would differ, but be dependent on the growing conditions in the experiment. Linked to this, the third objective was to explore the effect of different microclimates on the behaviour of AM fungi under conditions of salinity stress, comparing controlled and field conditions. Here, I hypothesised that field experiments would not reproduce the ideal growth conditions seen in a Constant Environment Room, and so mycorrhizas would appear to be less effective in the field.

Chapter 2

General Materials & Methods

2.1 Controlled experiments

Experiments were carried out under controlled conditions either in a controlled environment room (CER) or glasshouse at Royal Holloway, University of London. The CER had a light/dark cycle of 16/8 hours, temperature of 20°C and an average relative humidity of 60%. Fluorescent light tubes provided 620 μmol m^{-2} sec^{-1} intensity at plant level. Experiments in the glasshouse were carried out from the middle of March to the end of August each year (2013). The range of Photosynthetically Active Radiation (PAR) in the greenhouse was between 16 and 300 μmol m^{-2} sec^{-1}. There were two types of supplementary lights in the glasshouse; these included: high pressure sodium lamps (HPS) (250w) and bulb type Osram or Phillips son-t plus. During the summer season, the experiments were conducted in the glasshouse with photoperiods of 16/8 hours light and dark.

2.1.1 Field experiments

Experiments were carried out on the campus of Royal Holloway, University of London. The site was fenced with galvanised wire netting up to 2 m high and 30 cm down into the earth to prevent rabbits and deer from disturbing the experiment. Field experiments usually started mid-May and ended in August (Summer 2012 and 2013).

2.1.2 Seed germination and transplantation

Seeds of the host plants used for second generation testing in all of the experiments were grown under laboratory conditions with a constant light source. Five healthy looking seeds were randomly selected for each treatment and placed in a 90 mm Petri dish on moist filter paper (Whatman No. 1, 85 mm). The filter paper was kept moist by adding distilled water for seed germination.

Seeds were sown in standard plastic seed trays filled with John Innes potting compost No.2 (John Innes Association, Reading, UK). Trays were kept in the CER to attain optimal growth conditions and were watered daily with normal tap water. When the plant seedlings reached the four leaf growing stage, each was transferred to an individual 110 mm square plastic pot filled with John Innes potting compost No. 2 for the next experiment.

2.1.3 Growth substrate for experimental plants

The substrate used for the controlled experiments was John Innes compost No.2. This commercial compost is used for general potting of vegetables and house-plants, and is a mixture of sand, loam and peat. Each cubic metre of the compost contains the following fertilizer: 0.6 kg lime stone, 2.4 kg hoof and horn meal, 2.4 kg superphosphate and 1.2 kg potassium sulphate. The compost was heat treated according to the supplier's instructions and was free from pests and pathogens.

2.1.4 Nutrient solution

An all-purpose concentrated plant food was used (Miracle-Gro, Scotts Company, Godalming, UK). The nutrient solution was diluted to half-strength and given once every two weeks to each plant during the experiments. The concentrated plant food contained all of the necessary nutrient elements for plant growth; its composition is given in Appendix A.

2.1.5 Pesticides

The 'Bug Clear Gun' (Scotts Company, Godalming, UK) was used for controlled experiments in the CER. The product contains Pyrethrins, which are natural compounds that affect aphids and whitefly. This spray was used at the first sign of attack and then used frequently as needed.

2.2 Arbuscular mycorrhizal fungal inoculum

2.2.1 The use of a commercial inoculum

The commercial AM fungal mixture was obtained from Symbio Ltd (Wormley, Surrey) and the inoculum was in the form of an inert clay powder. The commercial mixture contains arbuscular mycorrhizal spores of *Glomus clarum*; *G. intraradices*; *G. mosseae*; *G. deserticola*; *G. monosporus*; *G. brasilianum* and *Gigaspora margarita*. Pots containing plants were inoculated with the commercial AM at a dose of 3 g per plant, with the powder placed in a layer underneath the roots to ensure maximum colonisation by the fungi. For the control plants, the commercial AM inoculum was treated with radiation by exposing it in a microwave oven at high temperature (not less than 100°C) for 4 minutes to ensure all spores were killed (Kahiluoto *et al.,* 2000).

2.2.2 The use of individual species of AM fungi

Two individual species of AM (*Glomus mosseae* and *G. etunicatum*) were obtained from Plant Works Limited (Innovation building, Kent Science park, Sittingbourne, Kent, ME9 8HL, UK). Approximately 1 litre of each species of mycorrhiza was obtained, consisting of small size gravels and root fragments. To maximise the quantities of AM species or fungal material available for experimental treatments, the AM species were bulk cultured using *Plantago lanceolata* as a host or 'trap' plant. Nine cm plastic pots were filled with John Innes compost no. 2 mixed with sand (50:50) to provide a low-nutrient condition, and approximately 9 g of each species of AM inoculum was placed inside the pot. Four to six *P. lanceolata* seedlings were planted in each pot, which was then placed inside a plastic Sunbag (Sigma Aldrich Company Ltd, The old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK). These are fitted with small holes for ventilation but reduce possible contamination. These bulk culture plants were kept in the CER and watered as needed with half strength nutrient solution supplied every 2 weeks. After 3 months of bulk culture propagation, the plants were harvested and roots with the growing medium were kept at a constant temperature of 4°C for future experiments. The success of this bulk culturing was verifed by taking root samples for AM colonisation quantification using a standard staining method (Vierheilig *et al.,* 1998).

2.3 Salinity treatments

Two types of salts were used in the controlled experiments. These were sodium chloride (NaCl) provided by Sigma-Aldrich and a mix of sea salt from Tropic-Marin Ltd (Dr. Biener GmbH, D-36367, Wartenberg, Germany). The manufactured sea salt contained all 70 trace elements found in natural seawater.

The plants treated with different species of AM fungi were established for up to 2 weeks before being treated with the first dose of salt. This was to ensure the establishment of AM colonisation and avoid sudden plant death due to salinity shock. Different experiments had different levels of saline electrical conductivity (EC) (illustrated in Appendix B). In all experiments conducted under controlled conditions the dose of salt solution used was 100 ml per plant pot each week. For experiments under field conditions, the salt dose was increased up to 200 ml each week per plant treatment.

Throughout the experiments, different levels of saline electrical conductivity (dS/m) were added to the plant treatments. Salinity stresses are typically categorised into three levels of salinity (Bernstein 1975): from 1 – 4 dS/m is considered a light saline effect on the plant, 4 – 8 dS/m is a medium salinity stress, and 8 dSm or more is deemed a high salinity level.

Weekly additions of salt solutions ensured that salts were not leached from the pots during daily watering, and that salt did not accumulate in the plant root zone to a higher dose than required.

2.4 Harvesting of experimental plants

After seed maturation, the plants were harvested. The measurements recorded for each plant included leaf number, inflorescence number, plant height, and inflorescence head length. The shoots were cut and labelled in paper bags and kept in an oven at 70°C for 3 days, after which final shoot biomass values were recorded. The roots of each plant were cleaned of soil or compost debris, labelled in plastic bags and kept in a freezer at –20°C for later AM fungal visualisation and quantification.

The seeds of each plant were cleaned manually; the outer layer of inflorescence was removed, the remaining inflorescence parts were discarded, and only the seeds were weighed. After cleaning, the seeds were kept in labelled paper bags to allow for air circulation to help maintain seed viability for next generation germination rate testing.

2.5 Visualisation and quantification of AM in host plants

2.5.1 Root clearing and staining

The staining and clearing protocol used for the root samples was taken from Vierheilig *et al.,* (1998). The roots were cleared from remaining debris under running water over a sieve so that the fine roots were kept intact. The fine roots were selected and cut into 1 cm pieces, then placed in a glass tube and labelled accordingly. For root clearing, potassium hydroxide (10%) was added to the test tube containing the root sample. The sample was then placed in a water bath at 80°C for 30 minutes. After the clearing step, the roots were rinsed several times with water and dried on tissue paper. The root sample was then placed in a second clean vial for the staining process. Acidic staining solution (84.4% distilled water: 15% (1 % HCL): 0.6 % Quink pen ink) was added to the vial and covered root sections. The tube was again placed in a water bath at 80°C for 15 minutes to speed up the staining process.

2.5.2 Quantification of AM fungal colonisation

AM fungal colonisation of the stained roots was quantified using the crosshair eye-piece method of McGonigle *et al*., (1989). Stained root pieces were placed on a glass slide under a coverslip. AM colonisation was visualised using a compound microscope at 100x magnification. One hundred intersections of the root pieces with the crosshair were counted and the different AM fungal structures were recorded to obtain the percentage of root length colonisation by the AM fungi. In addition, the three main structural components of the AM fungus, hyphae, vesicles and arbuscules, were recorded separately to understand the physiology and behaviour of the fungi under different stress regimes.

2.6 Plants species used in the experiments

2.6.1 Plantago lanceolata

Plantago Lanceolata (L.) is a perennial plant species with the common name of ribwort plantain that can be found in disturbed areas and is considered a weed in many countries (Tonsor *et al.*, 1992). Seeds germinate during the spring, with less than 1% of seeds germinating in summer or autumn, while most of the flowering occurs during July (Tonsor 1987). *P. lanceolata* is a wind pollinated plant and has a gametophytic stage in which each of its cells contains a single set of chromosomes (Ross 1973). Each inflorescence carries multiple seeds (Primack & Antonovics 1981). One ecological advantage of this plant is the low death rate recorded during the growing season and the large number of viable seeds produced (Mook *et al.*, 1989). It is classified as a strongly mycorrhizal plant and for this reason it has been used in many experiments targeting AM fungi (Orlowska *et al*., 2002).

Seeds of *P. lanceolata* used in the present experiments were obtained from Herbiseed Company (New Farm, Mire Lane, West End, Twyford, Berkshire, RG10 0NJ, UK), using the 2009 plant production stock.

2.6.2 Common sowthistle (Sonchus oleraceus)

The common name of this species is common sowthistle and it belongs to the Asteraceae family. *S. oleraceus* is an annual herb and can grow up to 110 cm tall. The seeds of this species are very light in weight and attached by white silky hair. It is very common around the world, but is native to Europe and North Africa (Holm *et al*., 1977). The plant species is identified as a weed in many countries and is usually found associated with crops, as well as on roadsides and gardens (Widderick, *et al*., 2004).

Seeds for this species were obtained from the Herbiseed Company, using 2009 plant production stock.

Chapter 3

Effect of arbuscular mycorrhizal fungi on offspring quality of *Sonchus oleraceus*

3.1 Introduction

Many factors determine plant seed production and seed viability for successful sibling or offspring propagation. Nutrient resource supply, diseases and herbivore attack are the most important elements controlling seed production and viability (Hendrix 1988; Lee 1988). In addition, mycorrhizal fungal associations with a plant can help to maximise nutrient resource supply and enhance resistance to diseases and herbivores (Koide 2010). There have been many studies investigating the ecological and physiological aspects of arbuscular mycorrhizal (AM) fungal interactions with plants. However, remarkably few have concentrated on the effect of AM fungi on plant reproductive attributes (Koide 2010). Limited research has focused on the effect of AM fungi on the reproductive system of a host plant, including studying seeds, flowers and the survival of second generation plants in relation to the parental plant treated with AM fungi.

Successful AM colonisation of certain plants can enhance the absorption of limited resources (especially phosphate) from soil and, through translocation, can benefit the reproductive system of the plant (Koide 2010). Some studies have confirmed the positive influence of AM colonisation through increasing nutrient absorption and plant reproduction in Indian mallow (*Abutilon theophrasti*) (Koide *et al*., 1994), tomato (*Lycopersicon esculentum*) (Bryla & Koide 1990) and wheat (*Triticum aestivum*) (Karagiannidis & Hadjisavva-Zinoviadi 1998). However, other studies showed different results even between the same species; for example, an association with AM fungi decreased seed production in wild oats (*Avena sativa*) but enhanced seed production in an agriculturally cultivated oat variety (Koide *et al.*, 1988b).

Further studies on the ability of different AM species to affect seed production have demonstrated that different species of AM have varying effects on particular plant species, leading to either an increase or decrease in seed germination. It has long been suspected that the variable effects of individual species of AM were due to variations in their ability to supply phosphate to the associated plant (Koide 2000). Cowpea (*Vigna unguiculata)* seed yield and nutrient composition were enhanced when the plant was

associated with *Glomus aggregatum*, *G. geosporum* and *Scutellospora calospora*, but seed production was far less in plants associated with *Acaulospora scrobiculata* and *G. sinuosum* mycorrhizas (Muthukumar & Udiayan 2002). Research on potted soybean (*Glycine max*) also indicated that association with *G. mosseae* resulted in improved reproduction and development compared to plants colonised by *Gigaspora rosea* and *G. etunicatum* (Bethlenfalvay *et al*., 1997).

The timing of AM colonisation of a plant also plays an important role in reproduction. For annual plants, the most positive results are obtained when the mycorrhizal association occurs before or during the reproductive stage (Koide 2010). In contrast, most perennial plants are enhanced by AM colonisation after reproduction. Mullen and Schmidt (1993) noted this in the Alpine buttercup (*Ranunculus adoneus*) during the Spring, when phosphate uptake is very difficult and the vegetative parts are growing.

As indicated by many studies, the association between AM and plants increases phosphate uptake. As a result of this, the time required for flowering is reduced, as shown in a study on *A. theophrasti* by Lu & Koide (1994). Hence, speeding up the process of flowering resulted in more flowers per plant, thus increasing seed production in plants colonised by AM fungi. It was estimated that AM colonisation of *A. theophrasti* could increase flower production by 64%, fruits by 24 % and significantly increase the number of seeds produced in each fruit by 16 % (Koide 2010). Also in *A. theophrasti*, the effect of AM fungi on different plant genotype associations did not alter the timing of flowering, yet still played a role in increasing seed production (Lu & Koide 1994). Furthermore, AM colonisation enabled the plant to attract more pollinating insects by increasing the size of the flowers, making them more attractive to insects and leading to greater seed production (Patton & Ford 1983). Moreover, it was reported that pollinator visits to flowers of African marigold (*Tagetes erecta*) were increased because the mycorrhiza helped in enhancing nectar quantity and quality (Gange & Smith 2005).

AM colonisation can also play a role in increasing seed weight by enhancing nutrient content. For example, mycorrhizas increased seed weight by 60% in wheat (*T. aestivum*) (Karagiannidis & Hadjisavva-Zinoviadi 1998), and seeds of wild oat (*Avena fatua*) associated with AM contained more phosphorus than seeds from plants without the association (Lu & Koide 1991). Thus, by increasing the content of phosphate and other nutrient elements like nitrogen in the seed, mycorrhizas can enhance seed

germination in the next generation (Koide & Lu 1992). Moreover, Shumway and Koide (1994) demonstrated that *A. theophrasti* colonised by mycorrhizas produced offspring with much better survival than untreated control plants.

Sow thistle (*Sonchus oleraceus* L.) is an annual plant species and can be found in many areas around the world (Xiong 1997). The uniqueness of this plant is that it occurs in disturbed sites where many other species cannot survive (Xiong *et al*., 1997). *S. oleraceus* has the ability to associate with a wide range of plant species; for example, in Southern Australia they found it was hosting *Helicoverpa armigera* (Walter & Benfield 1994). The seeds of sow thistle are easy to germinate and can be used as a model for germination behaviour.

In the present study, the aim was to examine the effect of inoculating AM fungi on sow thistle (*Sonchus oleraceus* L.) offspring growth. I hypothesised that using species of AM fungi would add many benefits to *Sonchus* growth performance such as vegetative growth and seed germination, and show a positive effect on offspring performance as well.

3.2 Materials and methods

Sonchus oleraceus seeds (described in section 2.6.2) were germinated in a controlled environment room (CER) (see section 2.1). At the four-leaf stage, 40 healthy plants were transferred to individual 11 cm square pots filled with compost (section 2.1.3). Twenty plants were treated with a commercial mix of AM fungi at root level (as described in section 2.2.1), and the other 20 *S. oleraceus* plants were grown without AM inoculation as an experimental control. The duration of the experiment was 4 months.

Seed production by first generation *S. oleraceus* plants from both the AM and the control treatments were collected at the end of the growth cycle and kept separately. Seeds produced from this first generation of *S. oleraceus* plants were propagated to produce 40 individual seedlings, 20 from the AM colonised parent plants and 20 from the control plants. All 40 seedlings were transferred to individual 11 cm square pots containing the same compost. Thus, a total of 40 *S. oleraceus* plants were tested for the effect of AM colonisation on the parental plants (first generation) and their effect on

offspring (second generation) under the same growing conditions in the CER (section 2.1). However, in the second-generation seedling test, AM fungal inoculum was not added to the roots, thereby forcing reliance on seed reserves from the first generation inoculation. The second-generation seedlings were allowed to grow for 4 months just as the first generation.

Growth parameters from both the first and the second-generation plants were recorded, including leaf number, plant height (to tip of shoot, leaf height not included), shoot water content and final shoot dry biomass. The roots of first generation *S. oleraceus* were cleared for staining (section 2.5.1) and the extent of AM fungal root colonisation was quantified (section 2.5.1).

The data obtained were tested for normality and then analysed by one factor Analysis of Variance (ANOVA) using the Unistat (version 6.0) statistical package. Tukey's test was used to separate the means of treatments involved.

3.3 Results

3.3.1 First generation S. oleraceus harvest.

Dry shoot biomass was significantly higher (approximately 20%) for control plants compared with plants treated with AM fungi (Table 3.1; Figure 3.1).

Table 3.1: Summary of results from Analysis of Variance of different plant growth parameters for *Sonchus oleraceus* plants either treated with a commercial arbuscular mycorrhizal fungi mix or not (control) at the first generation stage. All degrees of freedom = 1, 38.

Parameters	AM	
	F-value	P-value
Dry shoot biomass	6.0	$< 0.01^{**}$
Final leaf number	0.02	0.9
Plant height	0.47	0.5
Shoot moisture content	0.639	0.4

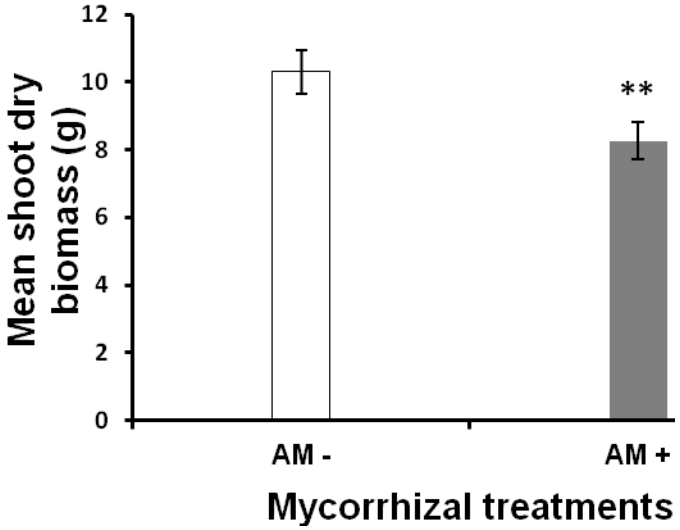

Figure 3.1: Final shoot dry biomass of *Sonchus oleraceus* plants colonised with the commercial arbuscular mycorrhizal fungi mix (AM+) and of control plants (AM-). Bars represent mean values (± SE), n = 20. **, statistically significant c.f. control, P < 0.01.

After four months of growth, the mean final leaf number did not differ between plants treated with AM fungi and the non-treated controls (Figure 3.2). A similar trend was observed for mean plant height; the application of the AM fungal inoculum had no effect on mean plant height at the end of the four-month growing period (Table 3.1; Figure 3.3).

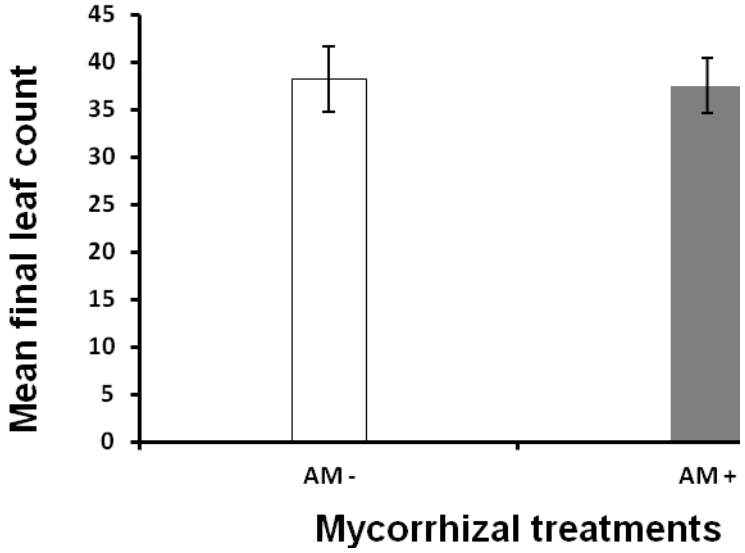

Figure 3.2: Final leaf number for *Sonchus oleraceus* plants colonised with the commercial arbuscular mycorrhizal fungi mix (AM+) and for control plants (AM-). Bars represent mean values (± SE), n = 20.

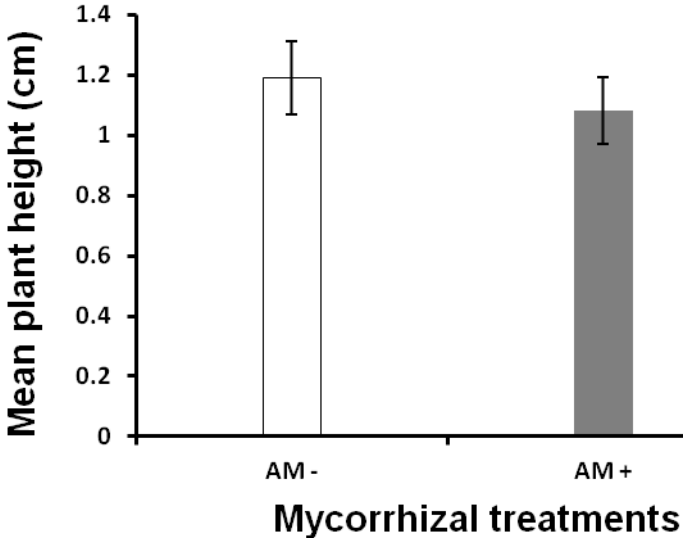

Figure 3.3: The height of *Sonchus oleraceus* plants colonised with the commercial arbuscular mycorrhizal fungi mix (AM+) and of control plants (AM-). Bars represent mean values (± SE), n = 20.

The analysis of shoot moisture showed that AM fungal inoculation had no effect on the mean water content of the plants (Table 3.1). The controls had slightly higher mean moisture content (23%) than the AM colonised plants (21%), but the difference did not reach significance (Figure 3.4).

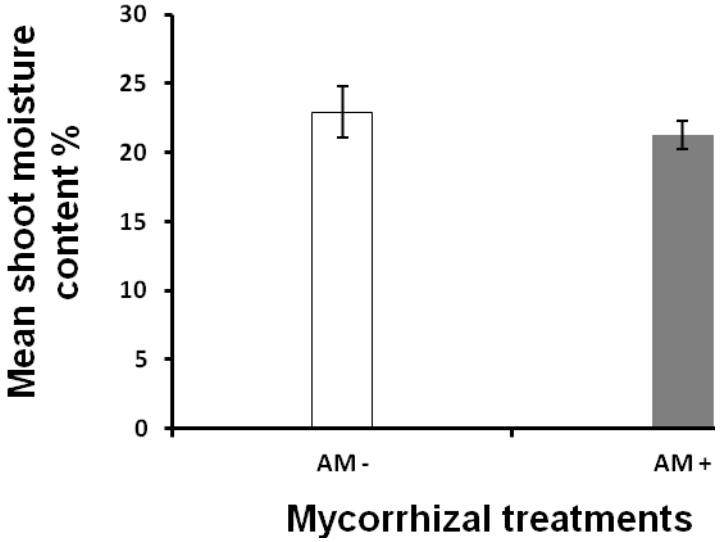

Figure 3.4: Shoot moisture content (%) of *Sonchus oleraceus* colonised with the commercial arbuscular mycorrhizal fungi mix (AM+) and of control plants (AM-). Bars represent mean values (± SE), n = 20.

Root staining data demonstrated successful colonisation by AM fungi in the treated plants (Table 3.2). On average, roots showed 40% colonisation by hyphae, 10% by vesicles and 4% by arbuscules. The control plants showed no incidence of AM fungal colonisation.

Table 3.2: Percent root length colonisation in *Sonchus oleraceus* plants treated with commercial arbuscular mycorrhizal fungi, or not treated (control) at the first generation stage.

	Hyphae		Vesicles		Arbuscules	
	Control	**AM**	**Control**	**AM**	**Control**	**AM**
Root colonisation (%)	0	40	0	10	0	4

3.3.2 Second generation Sonchus oleraceus harvest

With respect to dry shoot biomass, interestingly there was no significant difference between dry shoot biomass in offspring generated from AM colonised plants and the offspring from control parental plants. Control treatment offspring had a mean shoot biomass just 3% higher than offspring from AM colonised parents (Table 3.3; Figure 3.5). It should be noted that there was a large amount of variation between plants, demonstrated by the large standard errors (Figure 3.5).

Table 3.3: Summary of results of Analysis of Variance of different plant growth parameters for the offspring of *Sonchus oleraceus* in relation to their parental plant and either treated with the commercial arbuscular mycorrhizal fungi mix or not (control). All degrees of freedom = 1, 38.

Parameters	**AM**	
	F-value	**P-value**
Dry shoot biomass	3.17	0.083
Final leaf number	2.08	0.158
Plant height	0.7	0.41
Shoot moisture content	0.72	0.4

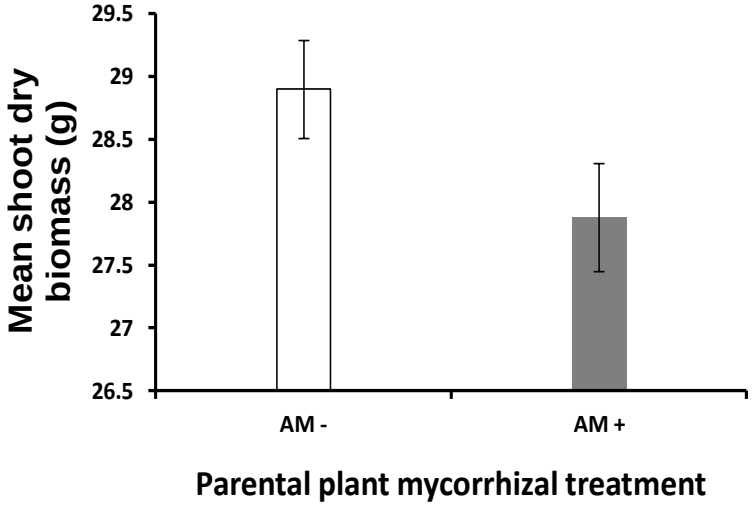

Figure 3.5: Dry shoot biomass of the offspring of *Sonchus oleraceus* plants whose parents were either colonised with the commercial arbuscular mycorrhizal fungi mix (AM+) or were control plants (AM-). Bars represent mean values (± SE), n = 20.

A similar trend was observed for total leaf number, which revealed that the offspring of parental plants associated with mycorrhizas had comparable mean leaf counts to the control (Table 3.3; Figure 3.6).

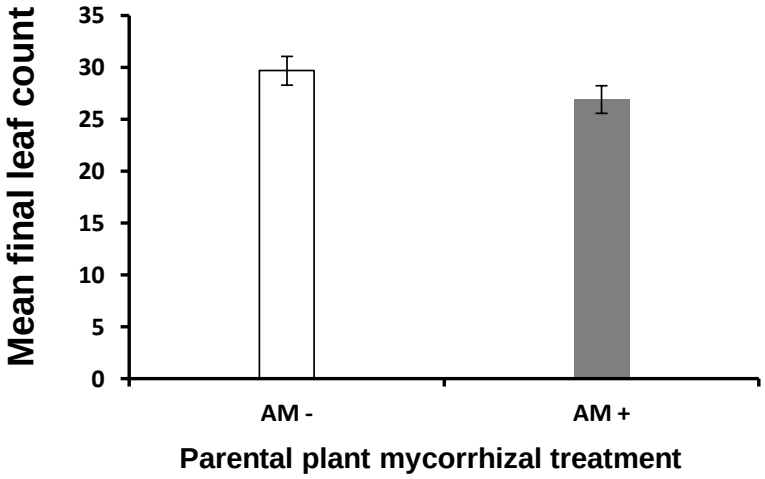

Figure 3.6: The final leaf number produced by second generation *Sonchus oleraceus* plants whose parents were either colonised with the commercial arbuscular mycorrhizal fungi mix (AM+) or were control plants (AM-). Bars represent mean values (± SE), n = 20.

Similar to the other growth parameters, mean plant height did not differ between plants grown from mycorrhizal or non-mycorrhizal parents (Table 3.3), even though the offspring of non-AM (control) parent plants produced slightly more offspring (7% higher) than the offspring of AM colonised parental plants (Figure 3.7).

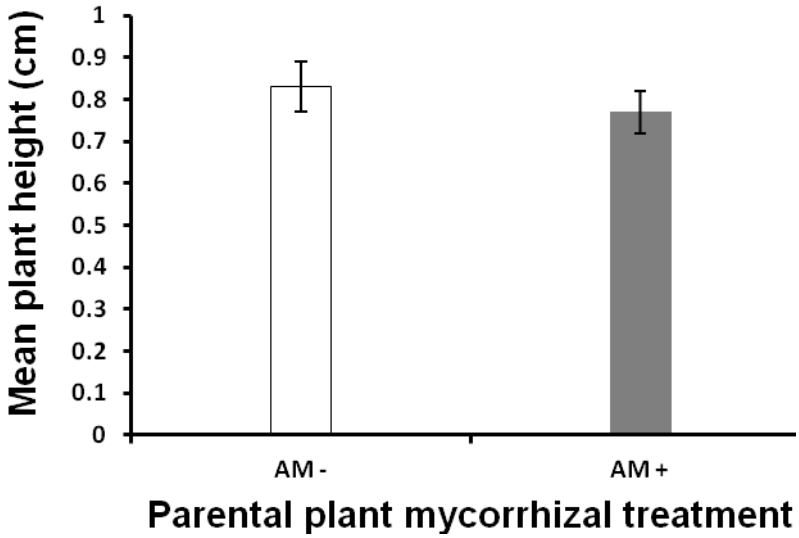

Figure 3.7: The height of second-generation *Sonchus oleraceus* offspring plants whose parents were either colonised with the commercial arbuscular mycorrhizal fungi mix (AM+) or were control plants (AM-). Bars represent mean values (± SE), n = 20.

Although the control plants contained 4% higher mean moisture content (Figure 3.8), the mean shoot moisture content in the offspring of the control parents was not significantly different from that recorded in the offspring of AM colonised parents (Table 3.3).

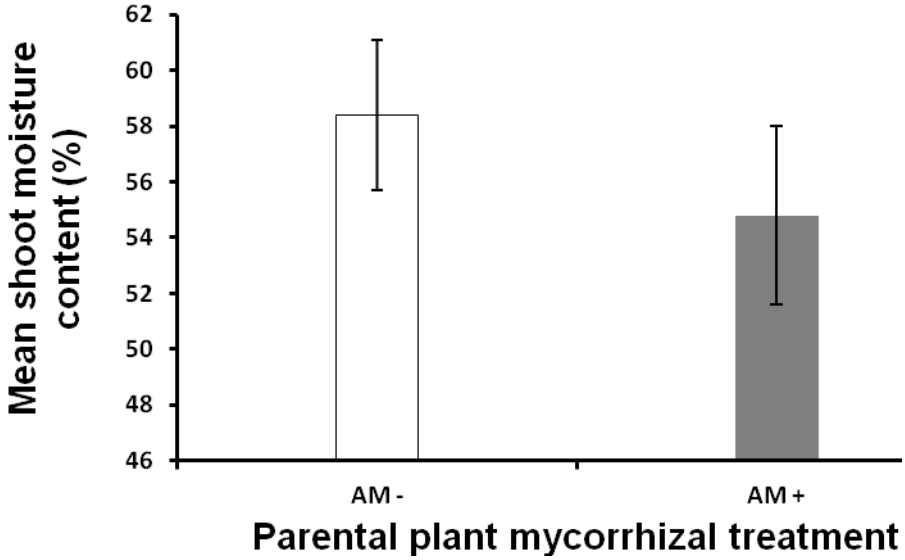

Figure 3.8: **The moisture content (%) of second generation *Sonchus oleraceus* plants whose parents were either colonised with the commercial arbuscular mycorrhizal fungi mix (AM+) or were control plants (AM-). Bars represent mean values (± SE), n = 20.**

3.3.3 Plant second generation morphology.

After allowing two weeks of seedling propagation for the second-generation plants (F2), the leaves of offspring plants not treated with mycorrhizas started to turn reddish brown as a sign of nutrient deficiency. On the other hand, seedlings of AM colonised parents did not exhibit any sign of stress or change in leaf colour (Plate 1).

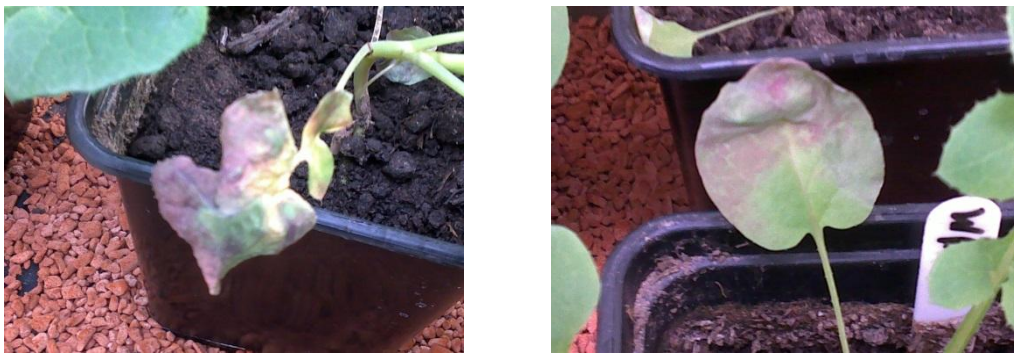

Plate 1: The stress colouration of small seedlings of second generation *Sonchus oleraceus* plants whose parents were not colonised by AM fungi.

3.4 Discussion

The findings presented in this study reveal that *S. oleraceus* plants with a mycorrhizal association did not demonstrate positive growth effects in comparison with control plants. Instead, the non-mycorrhizal *S. oleraceus* displayed a greater shoot biomass in the first generation. This lack of growth promotion with AM fungi manifested in the reduced biomass might indicate that the association between a plant and AM fungi can be negative and affect growth parameters adversely (Johnson *et al*., 1997). The previously reported growth retardation resulting from the addition of multiple mycorrhizal species to harebell plants *Campanula rotundifolia* (L.) (Nuortila *et al*., 2004) mirrors the result observed here. These authors stated that *Campanula* sp. displayed reduced total plant biomass when associated with three species of mycorrhizas (*G. boi, G. claroideum* and *Glomus sp*). In agreement, similar results were obtained in another study on the behaviour of different AM fungi species on *Anthoxanthum odoratum* and *Rumex acetosella* L., which showed that mycorrhizal plants displayed lower biomass than that of controls (Reynold *et al*., 2006).

On the other hand, in the current study, other growth parameters, namely plant height, leaf number and shoot moisture, were not significantly affected by the addition of mycorrhizas, which is consistent with previous findings in *Fragaria moschata* L. (Sudova & Vosatka 2008). This study indicated that inoculation with mycorrhizas had no influence at all in *Fragaria* compared to non-inoculated plants (Sudova & Vosatka 2008). Accordingly, the absence of a growth promoting effect or the negative influence of mycorrhizal association on plant growth was not surprising and is convergent with findings reported in previous studies.

One reason for the reduction in plant growth when they are associated with mycorrhizas might be related to the carbon demand of the fungi upon the host plant. In some situations the amount of carbon obtained by fungi from the plant root becomes larger than the benefits of the phosphorus provided to the plant (Johnson *et al*., 1997). Furthermore, a study on carbon drain from wheat (*T. aestivum*) in association with different AM fungi by Li *et al*. (2008) concluded that the identity of the AM fungal species, the type of plant it is associated with and the growth conditions all determine the extent of carbon losses by plant roots. As a rule of thumb, AM association costs a plant between 5 and 20% of its carbon. It may be that in *S. oleraceus,* as with other plants, the cost of the carbon drain by the AM fungi exceeded the 20% limit, causing

plant organ growth to slow down (Johnson *et al*., 1997). A study by Landis and Fraser (2008) gave another explanation: that carbon and phosphorus do not transfer at the same rate but depend on the demands of plant organs. Hence, according to the Landis and Fraser (2008) theory, at different points during plant growth the AM fungi can be either parasitic or beneficial depending on the growth situation and level of development of different plant organs.

S. oleraceus is classified as a vigorous weedy plant associated with crops around the world (Osten *et al*., 2004). It has been shown that AM fungi reduce the growth of weedy plants (Jourdan *et al*., 2000). For example, the addition of mycorrhizas significantly suppressed *Chenopodium album* and *Echinocloa crus-galli* L. weeds (Rinaudo *et al*., 2009). Moreover, in a study on enhancing sunflower production, the ability of mycorrhizas to suppress the growth of weedy plants was demonstrated (Rinaudo *et al*., 2010). The authors proposed two mechanisms that render the AM fungal association negative on weedy plants. The first mechanism directly affects the weed and the second mechanism affects it indirectly (Rinaudo *et al*., 2010). The direct effect includes the secretion of toxic compounds by AM fungi (Francis & Read 1995). The indirect mechanism, on the other hand, involves blocking the uptake of nutrients by weeds and diverting the transport of elements essential for growth to the non-weedy plant. Furthermore, AM fungi drain carbon from the weed roots and do not substitute it back for plant growth. The results of root staining in this experiment showed that only 4% of the root of *S. oleraceus* contained arbuscules, and it is known that reduced arbuscule formation by AM fungi usually happens in association with weedy plants (Rinaudo *et al*., 2010). For these reasons, it is tempting to speculate that the reduction in dry biomass is probably due to the weedy life style of *S. oleraceus* (Guil – Guerrero *et al.*, 1998).

In addition, the association of non-mycotrophs or facultative mycotrophic plants with AM fungi is believed to have either a negative effect on growth rate or no effect at all (Allen *et al*., 1989). More precisely, from field studies, reducing the activity of mycorrhizal fungi in the soil by applying fungicide did not negatively affect *S. oleraceus* populations or their growth, which means sow thistle is not mycorrhizal-dependent and can have improved growth without AM fungi (Gange *et al*., 1990). Another field study showed that *S. oleraceus* infesting crops had increased mycorrhizal colonisation, but this did not have any effect, either negative or positive, on its growth (Stejskalova 1989). Thus, the lack of effect of mycorrhizas on other growth parameters

(leaf number, plant height and shoot moisture content) in *S. oleraceus* classifies it as facultative-mycotrophic when treated with mycorrhizas.

In the present experiment a consortium of AM fungi species was used, which resulted in a decrease in one of the plant growth parameters, the dry shoot biomass. Previous experiments using multiple species of AM fungal colonisation also produced a negative effect rather than a positive one (Yocom 1985). Edathil *et al.* (1996) showed that adding *G. aggregatum* and *G. fasciculatum* to tomato (*S. lycopersicum*) decreased growth, especially shoot biomass. It was found that some species of mycorrhizas such as *Glomus* species might compete with each other instead of adding benefits to the plant (Hepper *et al*., 1988). An experiment on three plant species *Hieracium pilosella* L., *Bromus erectus* Huds. and *Festuca ovina* L. with four individual AM fungi or a mixture of them indicated different results, as some species gave a negative result with the mixture rather than with single mycorrhizas (Van der Heijden *et al*., 1998). It was suspected that multiple mycorrhizas can compete vigorously for sources of carbon instead of supplying it to the plant, or a mix of different mycorrhizas may disturb root function (Jansa *et al*., 2007). The reduction in shoot growth in *S. oleraceus* in first generation plants and the absence of a positive effect on other plant growth parameters conferred by root colonisation by multiple AM fungi is not in agreement with the Functional Complementarity theory proposed by Koide (2000). This theory states that different species of AM provide additional benefits for the plant when mixed together. Thus, our results on second and first generation plant growth parameters that favour the non-mycorrhizal treatment do not support the Functional Complementarity theory; instead, the data indicate that mixed species of AM had no effect on the tested growth parameters and only reduced the first generation dry shoot biomass.

It is known that the addition of mycorrhizas can enhance the protein and nutrient content of the seeds, conferring better establishment of second-generation plants (Elsheikh & Mohamedzein 1998). Also several studies have confirmed that the association of a plant with mycorrhizas enhanced reproduction and plant offspring (Lewis & Koide 1990; Lu & Koide 1991). On the other hand, another study showed the opposite results, where an association with AM fungi did not enhance seed production by wild oat (*A. fatua*) (Koide *et al*., 1988b). A further study on the effect of several species of AM fungi on cowpea (*V. unguiculata)* revealed that *A. scrobiculata* and *G. sinuosum* did not affect seed production (Muthukumar & Udaiyan 2002). Meanwhile, a study on soybean (*G. max*) seed quality in the context of association with different

species of AM (*G. mosseae*, *G. etunicatum* and *Gi. rosea*) gave different values for nutrient concentration and seed quality depending on the identity of the AM (Bethlenfalvay *et al*., 1997). The main reason for the diversity of effects of mycorrhizas on plant reproduction is due to the differing abilities of various AM species to enhance certain plants with phosphorus (Koide 2000). The lack of a positive effect of AM on the second generation of *S. oleraceus* in this study does not contradict the previous studies.

Plate 1 shows clearly changes in leaf colours in second-generation plants not colonised by mycorrhizas. At the four-leaf development stage the colour changed to red-purple as a sign of nutrient stress, probably phosphorus depletion (Wong 2005). In contrast to other growth parameters in the second generation that were not affected by the addition of mycorrhizas, the offspring (at the same growth stage) of *S. oleraceus* from parents colonised by AM fungi were healthy looking and showed a normal green colour without any signs of stress. It was reported in previous studies that mycorrhizal plants produce more vigorous and competitive offspring by supplying more phosphorus and other important growth elements (Koide 2010). It was proposed that by providing more nutrients to the plant, colonisation by AM fungi produces greater seed mass with larger concentrations of nutrient elements and necessary amino acids in the seed reserve, which can support offspring in the next generation (Bruckner *et al*., 1998; Oikeh *et al*., 1998). Likewise, inoculation of pea plants (*Pisum sativum* L.) with *G. mosseae* produced seeds with larger amounts of proteins and lipids at particular rates of soil phosphorus (Bethlenfalvay *et al*., 1994). The observation of stressed leaves reported in this study agrees well with the study of Bolland and Paynter (1990), suggesting that seeds with higher levels of phosphorus and other nutrient produce better germination and a higher concentration of phosphorus in their tissues. Furthermore, a field study on yarrow (*Achillea millefolium)* where mycorrhizas were eliminated by applying fungicide, produced seeds with reduced germination success compared with seeds produced from mycorrhizal plants (Allison 2002). Working on the effect of maternal association with AM on offspring quality, Koide and Lu (1995) proposed another mechanism by which *A. theophrasti* plants produce better offspring when they are associated with AM fungi. These authors proposed that mycorrhizal parents produce next generation plants that have the ability to initiate a growing root system faster, with a higher rate of phosphatase activity, aiding in greater absorption of soil nutrients.

3.5 Conclusion

The current findings reflect the complexity of the AM-plant association and the wide spectrum of effects emerging from this symbiosis. In *S. oleraceus*, association with mycorrhizas produced no stimulatory effect on the growth of the parental plants and their offspring. However, the morphological appearance of the second generation plants might point toward a beneficial effect of AM colonisation, possibly by accumulating more phosphorous and other nutrients in *S. oleraceus* and their offspring. Further future studies are needed to confirm this suggestion.

For the next experiments, it was decided to use a mycotrophic plant species that has the ability to exhibit positive results from AM associations during the first generation. This would allow for the examination of results on the offspring. Also, it may be better to monitor and record the first five days of seed germination of the next generation. This would result in a better understanding of the negative or positive effect of AM fungi on seed germination in the next generation of plants.

Chapter 4

Effect of salinity on arbuscular mycorrhizal colonisation of *Plantago lanceolata*, growth and offspring germination

4.1 Introduction

Soil salinity is one of the major challenges facing agricultural production worldwide – it has been estimated that one-third of agriculturally productive areas are damaged by soil salt accumulation (Kaynak *et al*., 2000). Salinity negatively influences plant growth and crop productivity (Ashraf & Foolad 2006; Ghazi & Karaki 2006). Each year, vast areas of arable land become unsuitable for good crop production or yield (Tuncturk *et al*., 2008). The Food and Agricultural Organization (FAO) estimated in 2005 that around 800 million hectares of land could not be used for agricultural productivity due to soil salinity (Munns 2005). In Turkey, for example, crop production on 32% of irrigated land is affected by high salinity (Ciftci *et al*., 2010). The major factors responsible for soil salinisation are irrigation and application of fertilisers (Epstein *et al*., 1980).

Soil salinity negatively affects plant growth and establishment by increasing the uptake of sodium and chlorine at the expense of other essential growth elements such as nitrogen and phosphorus (Saqib *et al*., 2006). Plant uptake of salts disturbs amino acid and carbohydrate production, which reduces the efficiency of the growth mechanism (Misra & Gupta 2005). Salt accumulation in the plant reduces stomatal turgor, thus decreasing photosynthesis and reducing carbon availability for the plant (Walker *et al*., 1981). For example, carbon allocation and sucrose metabolism were affected negatively when plants were grown under saline conditions (Balibrea *et al*., 2003). The osmotic balance faces severe disruption during salinity stress, preventing sufficient amounts of water from reaching the cells (Dorais *et al*., 2001). Also, growth regulators, such as gibberellic acid, are substantially reduced in the plant under salt stress, thereby reducing the rate of growth (Khan & Rizvi 1994). Furthermore, secondary metabolites can play an important role in the reaction of plants to salt stress (Senaratna *et al*., 2003). Phenolics and anthocyanins in particular were found to bind to toxic ions during salt toxicity thereby reducing cellular damage (Taiz & Zeiger 2002; Winkel-Shirley 2002). It was concluded that the main mechanism that helps halophytes to resist salinity stress even at high levels is by increasing the amount of secondary metabolites in the tissues

(Ksouri *et al.,* 2007). However, at high salinity levels, the secondary metabolites in many plant species are unable to combat salinity (Jain & Selvaraj 1997; Nelson *et al*., 1998). In sugarcane, for example, it was found that phenolics, anthocyanins and flavones were reduced in plants sensitive to salinity and had no effect on the ability of plants to cope with salt stress (Abdul & Ghazanfar 2006).

Arbuscular mycorrhizas have been known to exist in many saline environments where they help plants to overcome salinity stress (Aliasgharzadeh *et al*., 2001). Mycorrhizal colonisation of several plant species has been reported in salt marshes (Sengupta & Chaudhuri 1990) and coastal salty areas (Rozema *et al*., 1986). A substantial body of evidence shows that AM fungi can aid plants in overcoming salinity stress (Yano-Melo *et al*., 2003; Rabie 2005). One of the mechanisms by which AM fungi assist plants is by increasing nutrient absorption (Asghari *et al*., 2005). Mycorrhizas help to maintain a balanced K^+/Na^+ ion concentration inside the plant tissues, which is important for protection under salinity stress (Giri *et al*., 2003). Additionally, higher chlorophyll content in the leaves of mycorrhizal plants can give protection against the side effects of salinity (Giri & Mukerji 2004). Furthermore, AM fungi enhance the synthesis of the amino acid proline, which helps in maintaining osmotic balance during stress (Delauney & Verna 1993). Mycorrhizas decrease the uptake of sodium and chlorine by the roots, and block them from reaching the shoots (Scheloske *et al*., 2004). AM fungi encourage plants to produce antioxidant enzymes to fight oxidative damage caused by salinity (Wu *et al*., 2008). Danneberg *et al*. (1993) showed that AM fungi increase the secretion rate of the phytohormone abscisic acid (ABA), which facilitates phosphate absorption from the soil and helps to overcome stress.

Several studies have been conducted on the use of AM fungi for fighting soil salinity and for the successful establishment of plants. Inoculating maize (*Zea mays* L.*)* plants under saline conditions with *G. mosseae* resulted in higher chlorophyll content, more soluble sugars and higher electrolyte concentration in the roots, which helped to overcome salt stress (Feng *et al*., 2002). Inoculation of banana plants (*Musa* sp. Cv. Pacovan) with *Acaulospora scrobiculata*, *G. clarum* and *G. etunicatum* resulted in higher salt tolerance and greater biomass and leaf area than the non-mycorrhizal controls (Yano-Melo *et al*., 2003). Using *G. mosseae* to inoculate tomato plants under saline conditions increased the rate of essential nutrient elements in the shoot, and fruit

yield was greater than non-mycorrhizal tomato (Al-karaki 2006). Meanwhile, Sannazzaro *et al.* (2007), working on *Lotus glaber* Mill. plants inoculated with *G. intraradices* and grown under salt stress, showed an increase in the rate of proline production. Proline is an organic osmolyte, which aids resistance to salinity stress by adjusting cellular osmosis (Ashraf & Folad 2007). Proline is also important in maintaining the integrity of cellular membranes and prevents free radicals from damaging the plants (Srinivas & Balasubramanian 1995). Importantly, proline sustains the $NADP^+$/NADPH ratio throughout the metabolism during the stress (Hare & Cress 1997). In addition, it is believed that proline disintegration during stress relief can help to generate more ATP for recovery (Hare *et al*., 1998).

At the species level, different AM fungi have different abilities to enable plants to overcome environmental stresses (Daei *et al*., 2009). For example, *G. mosseae* was more effective in overcoming salinity stress in orange plants (*Citrus tangerine* Hort. Ex Tanaka) than *G. versiforme* (Wu *et al*., 2010). In this study, *G. mosseae* increased plant height, stem diameter, leaf number and decreased oxidative effects on the plant during salt stress far more than *G. versiforme* under the same conditions. Furthermore, differences in the genetic makeup of the AM species can alter their function in the plant association (Kuhn *et al*., 2001). Oliveira *et al*. (2010) studied the effect of different strains of *G. geosporum* obtained from different environments and found that strains from saline soil were better adapted to help plants overcome salinity stress than those from non-saline soil. Whether to use a mix or individual species of AM fungi to overcome the stress has also been debated. It was recommended that using a mix of species of mycorrhizas for the same plant would give better results than using only individual AM species (Alkan *et al*., 2006). Using a mix of *G. mosseae*, *G. claroideum* and *G. intraradices* as inoculum for *Astragalus sinicus* L. plants under different salinity levels gave higher resistance to the effects of salt on plant growth than using individual inoculations of the same AM species (Peng *et al*., 2011).

Seed germination is not only influenced by environmental factors and the surrounding habitat, but also by the conditions in which the parental plant grew, which affects the quality and germination of the offspring seeds (Rossiter 1996). Thus, the phenotype of offspring changes to cope with the environmental stresses to which their parental plants were exposed (Donohue 2009). In angiosperm plants, around two-thirds

of genetic makeup and DNA composition that the environment influenced in the mother plant passed to the seeds (Mazer & Gorchov 1996). Moreover, environmental factors influence pollen quality and quantity in the parent plant (Delph *et al.*, 1997). There have been a few studies on the effect of mycorrhizas on second-generation plants, but almost no experiments have been done on the effect of salinity stress and mycorrhiza inoculations on seed production and second-generation seedlings.

The aim of this study was to investigate the effect of AM fungi on *Plantago lanceolata* plants and their second-generation seedlings under salinity stress. Specifically, the objectives were: (1) to examine the ability of mycorrhizas to overcome different levels of salinity stress; (2) to determine the interaction of mycorrhizas with different salt types (mixed salts and NaCl); and (3) to explore the effects of mycorrhizas on plant offspring quality. Thus, the first hypothesis was posed to address the question of whether mycorrhizas can alleviate salinity stress, and the second hypothesis was intended to test the dependency of mycorrhizal effects on salt type. Unfortunately, these hypotheses could not be adequately tested because mycorrhizal colonisation could not be confirmed at the end of the 4-month experiments for reasons beyond my control. Therefore, the mycorrhizal effect has been omitted from the discussion (though remains in the analysis) and the aims were narrowed to focus only on the effect of different salinity stress levels induced by a single salt and mixed salts on *Plantago lanceolata*. The problem of colonisation failure was, however, overcome in the next chapter.

4.2 Materials and methods

4.2.1 Experiment 1: NaCl salt

A total of 32 plants were selected for this experiment and inoculated with commercial AM fungi (section 2.2.1). Four levels of salinity treatment were used and each contained eight replicates. In this experiment, all of the plant replicates were treated with AM fungi and there were no non-mycorrhizal controls due to a shortage of space in the CER (a major limitation) and because the aim was to determine if salt affected AM inoculum performance. Thus, in the first experiment with NaCl salt addition, all of the plant replicates were treated with AM fungi and there was no control.

Plantago lanceolata (section 2.6.1) seeds were germinated in the CER (section 2.1). After the seedlings reached the four-leaf stage, the healthy-looking seedlings were transferred to individual 11-cm square pots filled with commercial sterilised compost (section 2.1.3).

The experiments were divided into four salt treatments of EC (electrical conductivity, dS/m): 2.2 dS/m (light), 5 dS/m (medium), 10 dS/m (high) and tap water as the control (Appendix B). Each week, 100 ml of salt solutions was applied to each treatment plant to keep the desired salinity level in each pot constant. At 2-week intervals, nutrient solution was added to the plants (section 2.1.4).

The experiment lasted for 4 months and after this time plant height, leaf number, and inflorescence number and length were counted. Also, the weights of inflorescences were recorded to give an indication of the weight of the seeds contained in each. Initial shoot biomass (fresh weight) was taken for each plant and final dry shoot weight to measure the final shoot biomass for each treatment (section 2.4). Roots of each plant were cleaned and stained for AM visualisation (section 2.5.1), quantification of colonisation and identification of different parts of the AM fungus such as vesicles, hyphae and arbuscules (section 2.5.2).

The seeds produced from F1 (parent) plants treated under the salinity conditions as described above were used for the germination of F2-generation plants. From each F1 plant, 15 healthy seeds for the germination test were selected. Petri dishes of 90-mm diameter with filter paper inside were used for the germination test under constant room temperature (26˚C). The 15 seeds of each plant were divided into three Petri dishes, so that five seeds were placed into each Petri dish giving a total of 15 seeds for each plant. The seeds were watered daily with distilled water and daily seedling germination was recorded for 7 days of the experiment, after which the final total shoot and root length for each successful germinated seedling was recorded.

This experiment employed one-way ANOVA (after normality testing) to determine the effect of different salt levels only, with no testing for a mycorrhizal effect as there was no control treatment. Data were analysed using the Unistat version 6.0 statistical package.

4.2.2 Experiment 2: mixed salts

A total of 48 plants were selected and mixed salts (section 2.3) were used to induce salinity stress. There were four salinity levels, each having six replicates inoculated with commercial AM fungi (section 2.2.1) and six as non-mycorrhizal controls (to overcome the limitation of experiment 1). Thus, half of the plants were inoculated with commercial AM fungi (section 2.2.1) at the root level to produce an AM-colonised treatment.

Plantago lanceolata (section 2.6.1) seeds were germinated in the CER (section 2.1). After the seedlings reached the four-leaf stage, the healthy-looking seedlings were transferred to individual 11-cm square pots filled with commercial sterilised compost (section 2.1.3).

The experiment was divided into four salt (electrical conductivity) treatments: 2.2 dS/m (light), 5 dS/m (medium), 10 dS/m (high) and tap water as the control (Appendix B). Each week, 100 ml of salt solution was applied to each treatment plant to keep the desired salinity level in each pot constant. At 2-week intervals, nutrient solution was added to the plants (section 2.1.4).

The experiment lasted for 4 months and after this time plant height, leaf number, and inflorescence number and length were counted. Also, the weights of inflorescences were recorded to give an indication of the weight of the seeds contained in each. Initial shoot biomass (fresh weight) was taken for each plant and final dry shoot weight to measure the final shoot biomass for each treatment (section 2.4). Roots of each plant were cleaned and stained for AM visualisation (section 2.5.1), quantification of colonisation and identification of different parts of the AM fungus such as vesicles, hyphae and arbuscules (section 2.5.2).

The seeds produced from F1 (parent) plants treated under the salinity conditions as described above were used for the germination of F2-generation plants. From each F1 plant, 15 healthy seeds for the germination test were selected. Petri dishes of 90-mm diameter with filter paper inside were used for the germination test under constant room temperature (26˚C). The 15 seeds of each plant were divided into three Petri dishes, so that five seeds were placed into each Petri dish giving a total of 15 seeds for each plant. The seeds were watered daily with distilled water and daily seedling germination was

recorded for 7 days of the experiment, after which the final total shoot and root length for each successful germinated seedling was recorded.

Data from this experiment were tested for normality prior to subjecting the data to factorial ANOVA, employing salt and AM colonisation as the main effects, and Tukey's test was used for means separation. The statistical package Unistat version 6.0 was used to analyse data. The results of the Analysis of Variance are summarised in tables and only the comparisons that showed statistically significant differences between subgroups are represented graphically.

4.3 Results

4.3.1 Experiment 1: NaCl salt

Most plant growth parameters were significantly affected by the salt treatments as summarised in Table 4.1. The highest salinity treatment (10 dS/m) resulted in shorter plants in comparison with the control plants only, while plant height was unaffected in the other salinity treatment groups (Figure 4.1).

Table 4.1: **Summary of the results from Analysis of Variance testing for the effect of different NaCl levels on plant growth parameters of *Plantago lanceolata* inoculated with commercial AM fungi. The degrees of freedom = 3, 28**.

Parameters	F-value	P-value
Plant height	3.35	< 0.05
Leaf number	14.66	< 0.001
Shoot dry biomass	12.7	< 0.001
Inflorescence number	6.96	< 0.001
Average inflorescence head length (cm)	2.19	0.111
Inflorescence head weight (g)	7.61	< 0.001

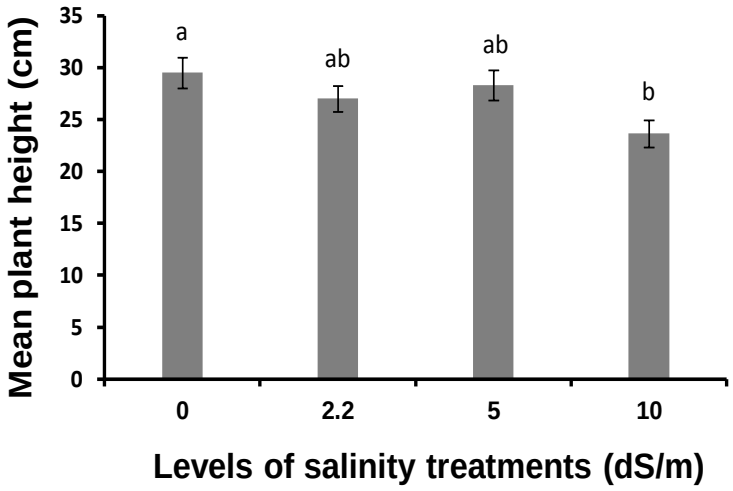

Figure 4.1: The height of *Plantago lanceolata* inoculated with commercial AM fungi under different levels of NaCl salinity (0, 2.2, 5 and 10 dS/m). Bars that are statistically similar share the same letter only, whereas different letters indicate a significant difference (P <0.05).

The other parameter that was significantly affected by NaCl salinity stress was the plant leaf number (Table 4.1; Figure 4.2). The highest salt level treatment (10 dS/m) produced the lowest leaf number in comparison with the control and with all the other salt treatments (Figure. 4.2). There was no significant difference between the control and the low (2.2 dS/m) and medium (5 dS/m) salinity treatments; however, the leaf number was significantly lower in the medium and high salinity treatments compared to the low salinity level treatment (Figure 4.2).

The addition of salt also significantly reduced the final shoot dry biomass (Table 4.1) but only at the highest level of NaCl addition (Figure 4.3). The low and medium salt treatments did not affect the shoot dry biomass compared with the control group, yet both salt treatments were significantly different from the highest salinity level group (Figure 4.3).

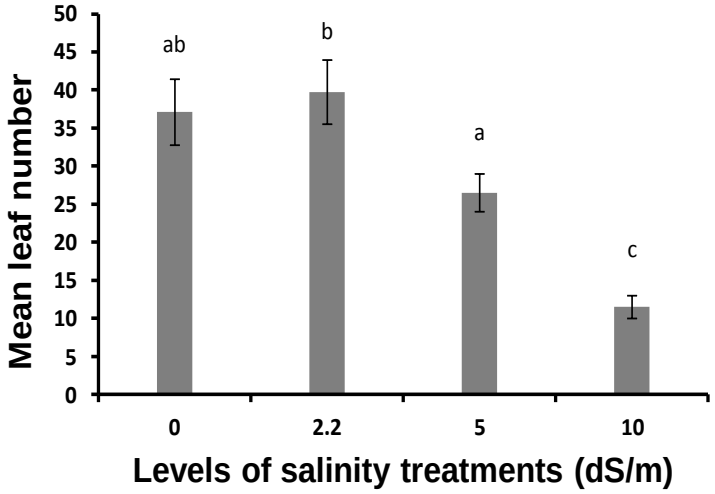

Figure 4.2: Leaf number of *Plantago lanceolata* inoculated with commercial AM fungi under different levels of NaCl salinity (0, 2.2, 5 and 10 dS/m). Bars that are statistically similar share the same letter only, whereas different letters indicate a significant difference (P <0.001).

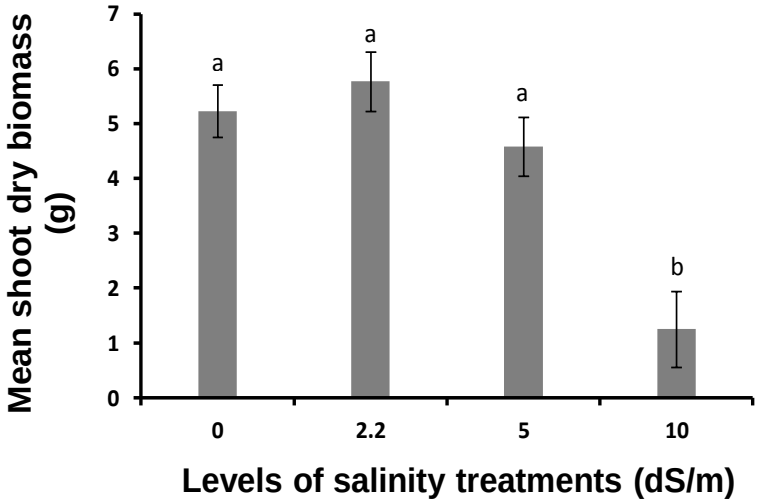

Figure 4.3: Final shoot dry biomass (g) of *Plantago lanceolata* inoculated with commercial AM fungi under different levels of NaCl salinity (0, 2.2, 5 and 10 dS/m). Bars that are statistically similar share the same letter only, whereas different letters indicate a significant difference (P <0.001).

Another parameter that was significantly affected by salinity stress was the inflorescence number (Table 4.1). The number of inflorescences per plant was markedly reduced under the highest salinity (10 dS/m) treatment (Figure 4.4). Initially, light salinity (2.2 dS/m) caused a minor increase of inflorescence number compared to the control but this difference was not statistically significant (Figure 4.4). On the other hand, there was no significant effect of salt addition on the average length of inflorescences (cm) (Table 4.1) hence the means separation test was not performed (graph not shown).

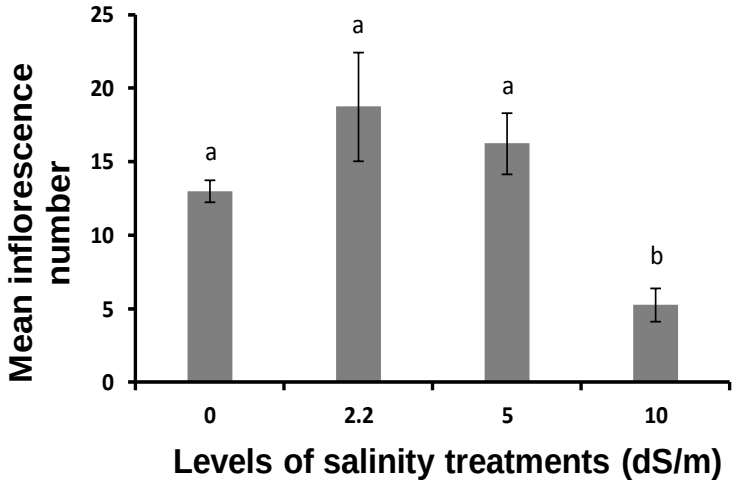

Figure 4.4: The production of inflorescence by *Plantago lanceolata* inoculated with commercial AM fungi under different levels of NaCl salinity (0, 2.2, 5 and 10 dS/m). Bars that are statistically similar share the same letter only, whereas different letters indicate a significant difference (P <0.001).

The inflorescence weight was also examined and demonstrated a significant difference with the salt treatments (Table 4.1; Figure 4.5). Plants treated with light (2.2 dS/m) and medium (5 dS/m) salinity produced higher inflorescence weight (Figure 4.5). Compared to the control plants, the highest NaCl treatment (10 dS/m) did not markedly affect the inflorescence weight (Figure 4.5); however, it reduced the inflorescence weight significantly in comparison with the medium and light salinity stress treatments, resulting in the lowest seed weight values. Thus, the addition of light and medium salinity to *P. lanceolata* enhanced the seed weight and flower component in the

inflorescences but this beneficial effect of salinity was diminished under the high salinity levels.

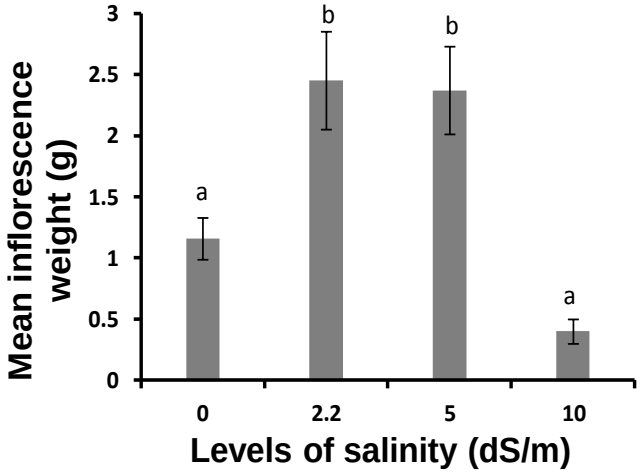

Figure 4.5: Inflorescence weight (g) of *Plantago lanceolata* inoculated with commercial AM fungi under different levels of NaCl salinity (0, 2.2, 5 and 10 dS/m). Bars that are statistically similar share the same letter only, whereas different letters indicate a significant difference (P <0.001).

The addition of salt had no significant effect on the seed germination parameters (seed germination percentage and seedling growth length) of *P. lanceolata* offspring as summarised in Table 4.2.

Table 4.2: Summary of the results from Analysis of Variance testing for the effect of salinity on offspring germination parameters of the second generation from parental *Plantago lanceolata* inoculated with commercial AM fungi under different levels of NaCl salinity. The degrees of freedom = 3, 188.

Parameters	F-value	P-value
Seed germination (%)	1.8	0.1
Seedling growth length (cm)	0.8	0.5

As shown in Figure 4.6, the percentages of germinated seeds were similar in all the salt-treatment groups. Likewise, measurement of the length of the offspring seedlings 1 week after germination did not indicate any significant effect of the addition of NaCl at different salinity levels. The mean seedling length from the medium salinity

treatment (5 dS/m) was modestly higher than the other treatments, whereas the highest salinity level seemed to slightly reduce the seedling length, though no statistical significance was detected (Figure 4.7).

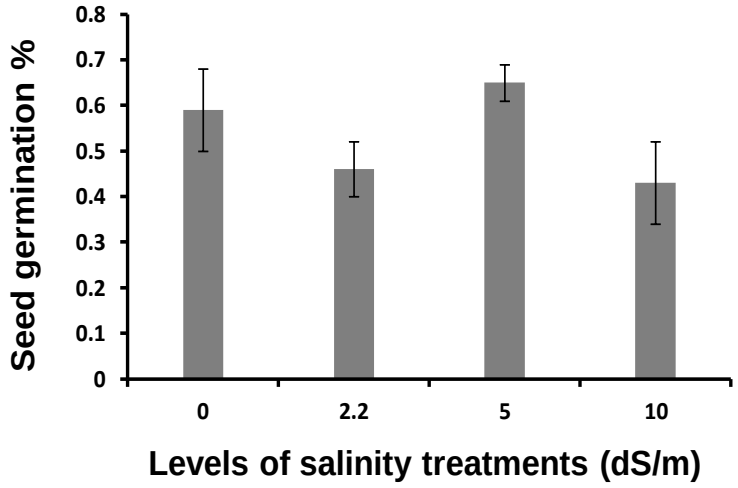

Figure 4.6: Seed germination of the second generation from parental *Plantago lanceolata* inoculated with commercial AM fungi under different levels of NaCl salinity (0, 2.2, 5 and 10 dS/m).

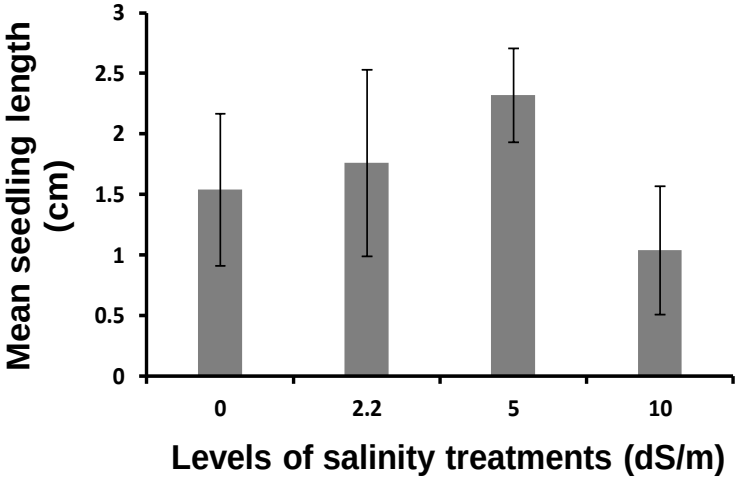

Figure 4.7: Seedling length of second-generation *Plantago lanceolata* plants after 7 days of germination. The parental plants were inoculated with commercial AM fungi under different levels of NaCl salinity (0, 2.2, 5 and 10 dS/m).

Importantly, there was no conclusive result confirming AM colonisation in the first-generation plants in any of the salinity treatments in experiment 1. At the end of

the 4-month experiment, AM structures could not be seen in the roots of any plant, which might be attributed to two possibilities: either the detection method was not sensitive enough to detect the AM fungi visually, or the fungi failed to effectively colonise the roots under our experimental conditions. The second possibility was more convincing and hence was considered in the interpretation of the findings. Additionally, the lack of a non-AM control, another limitation of this experiment, made it inappropriate to test the ability of AM to overcome salinity stress. Therefore, the aim of experiment 1 was modified and limited to test the effect of NaCl salt stress on *P. Lanceolata* inoculated, but not colonised, with AM.

4.3.2 Experiment 2: mixed salts

In this experiment, plant growth parameters and second-generation germination were studied in *P. lanceolata* inoculated with AM fungi and exposed to different levels of salinity using mixed salts. The effects of the mixed salts, AM fungi and the interaction of these two factors were examined and a summary of the statistics is shown in (Table 4.3).

Table 4.3: Summary of the results from Analysis of Variance testing for salinity effects on different plant growth parameters of first-generation *Plantago lanceolata* plants. Salt salinity levels are represented by EC (2.2, 5, 10 dS/m). AM is arbuscular mycorrhizal inoculation. The degrees of freedom for salinity = 3, 40; for AM = 1, 40; for the interaction term = 3, 40.

Parameters	Salts		AM		Salts x AM	
	F-value	P-value	F-value	P-value	F-value	P-value
Plant height (cm)	9.9	< 0.001	0.49	0.49	2.3	0.9
Leaf number	9.9	< 0.001	0.51	0.48	1.5	0.23
Dry shoot biomass (g)	2.4	0.08	1.1	0.31	0.49	0.69
Inflorescence number	13.1	< 0.001	0.95	0.34	0.03	0.99
Inflorescence head length (cm)	1.7	0.18	0.07	0.8	0.4	0.63
Seed weight (g)	15.1	< 0.001	1.9	0.18	0.66	0.58

Plant height was significantly reduced by salt exposure, whereas the treatment with AM fungi alone or in combination with salts did not apparently affect plant height (Table 4.3). Specifically, the reduction of plant height was significant at the highest salt level of 10 dS/m (Figure 4.8).

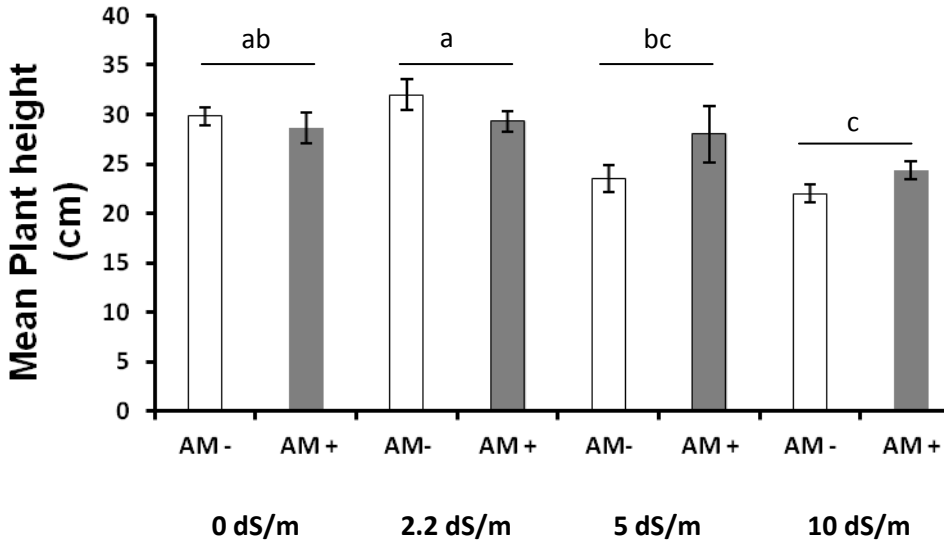

Figure 4.8: The height (cm) of *Plantago lanceolata*, with or without commercial AM fungi inoculation, under different levels of mixed salts. Shaded bars represent plants inoculated with AM fungi, and white bars indicate non-mycorrhizal plants. Groups that are statistically similar with respect to salinity effect share the same letter only, where as different letters indicate a significant difference (P <0.001).

The addition of AM fungi alone or in combination with the salts did not significantly affect the leaf-number parameter (Table 4.3). However, leaf number was significantly diminished in *P. lanceolata* exposed to mixed salts alone (Table 4.3; Figure 4.9). Under medium (5 dS/m) and high (10 dS/m) salinity stresses, leaf number was reduced significantly in comparison with the control (Figure 4.9). Meanwhile, there were no significant effects of the different salinity treatments or the addition of AM fungi on final shoot dry biomass (Table 4.3, graph not shown).

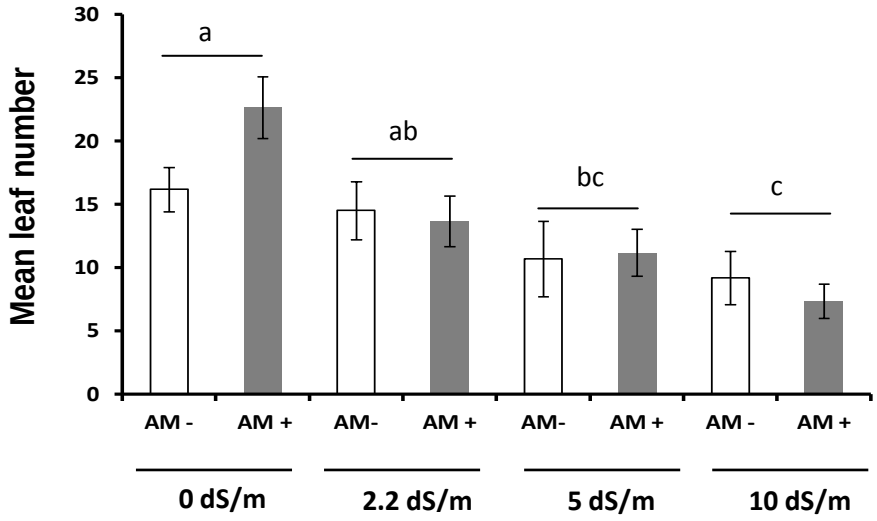

Figure 4.9: Plant leaf number of *Plantago lanceolata*, with or without commercial AM fungi, under different levels of mixed salts. Shaded bars represent plants inoculated with AM fungi, and white bars indicate non-mycorrhizal plants. Groups that are statistically similar with respect to salinity effect share the same letter only, whereas different letters indicate a significant difference (P <0.001).

As encountered with plant height and leaf number, salinity stress markedly diminished the inflorescence number of *P. Lanceolata*, with no apparent effect of the AM fungi addition on this parameter (Table 4.3). Increasing the salinity level from low (2.2 dS/m) to high (10 dS/m) was concomitantly associated with a sharp decline in inflorescence number (Figure 4.10).

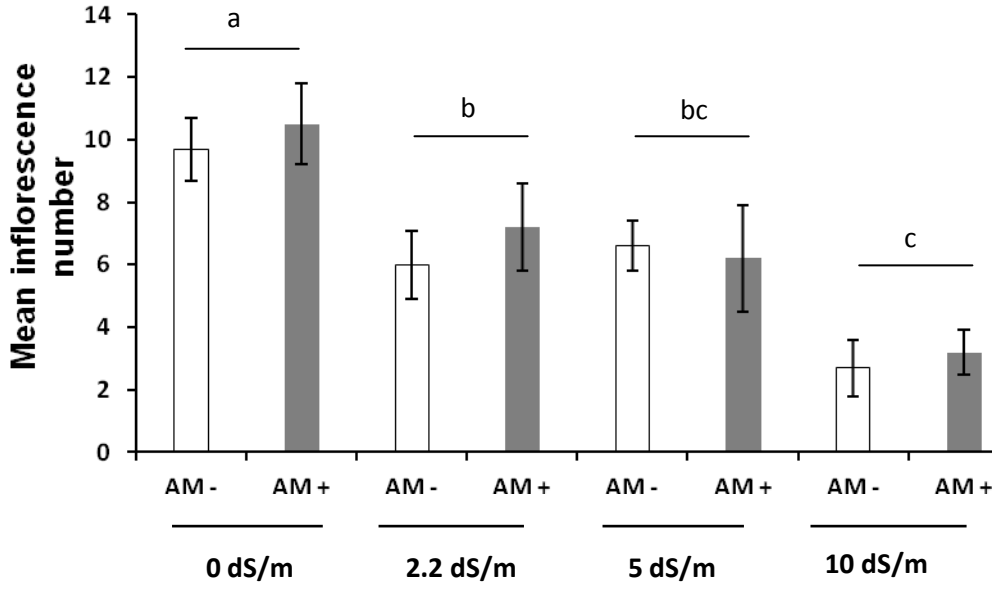

Figure 4.10: Inflorescence number of *Plantago lanceolata*, with or without commercial AM fungi, under different levels of mixed salts. Shaded bars represent plants inoculated with AM fungi, and white bars indicate non-mycorrhizal plants. Groups that are statistically similar with respect to salinity effect share the same letter only, whereas different letters indicate a significant difference (P <0.001).

With respect to the inflorescence head length, neither the addition of mixed salts nor the inoculation with AM fungi produced significant differences (Table 4.3, graph not shown). However, the addition of mixed salts, but not AM, significantly reduced another parameter, *i.e.* the seed weight (Table 4.3; Figure 4.11). The smallest seeds were produced at the highest salinity treatment (Figure 4.11). There was a suggestion that AM fungi could ameliorate the effect of salt at intermediate salinity levels only, but no significant interaction term was found between salt and AM in this experiment (Table 4.3).

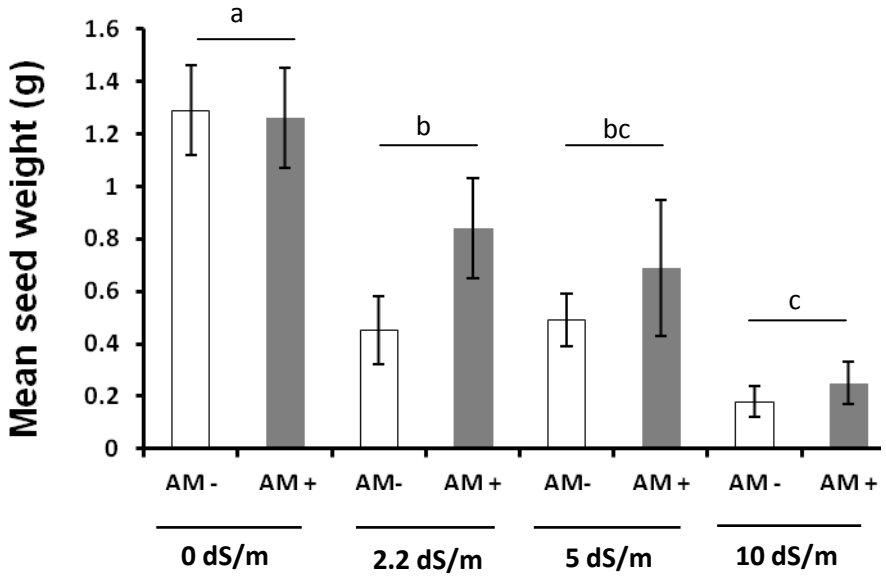

Figure 4.11: Seed weight (g) of *Plantago lanceolata,* inoculated with commercial AM fungi or not, under different levels of mixed salts. Shaded bars represent plants inoculated with AM fungi, and white bars indicate non-mycorrhizal plants. Groups that are statistically similar with respect to salinity effect share the same letter only, whereas different letters indicate a significant difference (P <0.001).

The effects of mixed salts treatment were also studied in the second-generation plants. Seed germination percentage and seedling length were significantly affected by mixed salts salinity stress but not by mycorrhizal addition (Table 4.4).

Table 4.4: Summary of the results from Analysis of Variance for second-generation *Plantago lanceolata* seedling germination when parental plants were inoculated with or without commercial AM fungi and were exposed to different levels (EC 2.2, 5, 10 dS/m) of mixed salts. Degrees of freedom for mixed salts = 3, 40; for AM colonisation = 1, 40; for the interaction term = 3, 40.

Parameters	Salts		AM		Salts x AM	
	F-value	P-value	F-value	P-value	F-value	P-value
Seed germination (%)	2.8	< 0.05	0.23	0.63	2.7	0.06
Seedling length (cm)	4.6	< 0.01	0.38	0.54	4.5	< 0.01

Mycorrhizal inoculation of parental plants did not enhance seed germination in the second-generation plants (Table 4.4). However, by increasing salinity level to 10 dS/m, the percentage of seed germination decreased significantly (Figure 4.12).

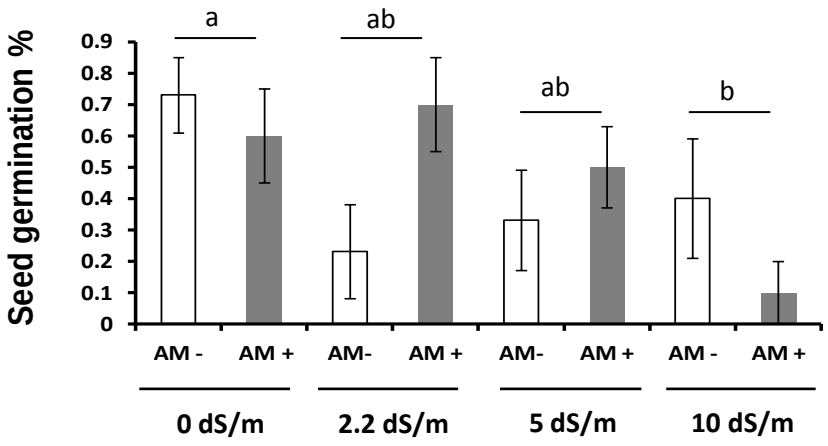

Figure 4.12: The percentage (%) seed germination of second-generation *Plantago lanceolata* when parental plants were inoculated with or without commercial AM fungi and were exposed to different levels (2.2, 5, 10 dS/m) of mixed salts. Shaded bars represent plants inoculated with AM fungi, and white bars indicate non-mycorrhizal plants. Groups that are statistically similar with respect to salinity effect share the same letter only, whereas different letters indicate a significant difference (P <0.05).

Seedling length decreased significantly as salinity stress on the parental plants increased (Figure 4.13). There was a significant decline in seedling growth when the parental plants were exposed to salinity levels of 10 dS/m (Figure 4.13). However, no reduction of seedling length was observed at the low and medium salinity levels (Figure 4.13).

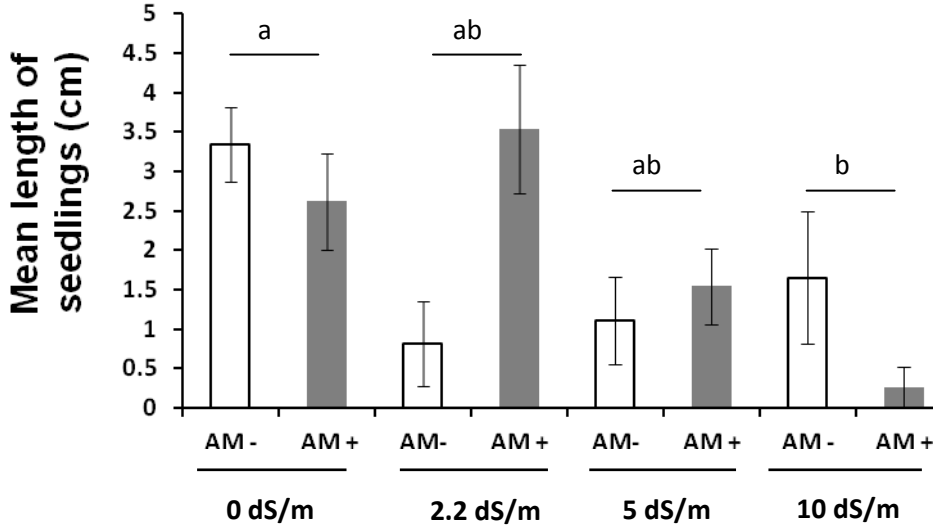

Figure 4.13: Length (cm) of second-generation *Plantago lanceolata* seedlings when parental plants were inoculated with or without commercial AM fungi and were exposed to different levels (2.2, 5, 10 dS/m) of mixed salts. Shaded bars represent plants inoculated with AM fungi, and white bars indicate non-mycorrhizal plants. Groups that are statistically similar with respect to salinity effect share the same letter only, whereas different letters indicate a significant difference (P <0.01).

At the end of this experiment, AM colonisation was not evident and no AM fungal structures were detected in the roots of the plants. Thus, the AM factor was not considered in the discussion.

4.4 Discussion

4.4.1 Experiment 1: NaCl salt

The findings from the first experiment revealed that exposing the plant to various concentrations of NaCl significantly affected several growth parameters. At high salinity levels (10 dS/m), vegetative growth sharply declined, as manifested by the diminished plant height and leaf number (Figures 4.1 and 4.2). A considerable body of evidence supports the devastating effect of salinity stress on plant growth. As such, it has been reported that under high salinity levels, leaf area tends to decrease, leading to a reduction in photosynthesis and carbohydrate production in wild radish (*Raphanus sativus* L.) plants (Marcelis & VanHooijdonk 1999). Another study showed that exposing potato plants (*Solanum tuberosum* L.) to high salinity stress changed the shape of the cells in the leaves and reduced intercellular spaces, resulting in a decreased chloroplast number (Bruns & Hecht-Buchholz 1990). Furthermore, higher salinity stress may be associated with problems in water retention in plant cells and negative osmosis (Hermandez *et al*., 1999; Meloni *et al*., 2001; Romeroaranda *et al*., 2001). Also, soluble proteins at higher salinity levels are reduced in plant tissue (Wang & Nii 2000; Parida *et al*., 2002). For example, salinity was shown to cause a reduction in protein content in peanut (*Arachis hypogaea* L.) (Hassanein 1999) and in maize (*Zea mays*) roots (Tamas *et al*., 2001). Lipids are another important element in plant cells that are severely damaged by high salinity (Kerkeb *et al*., 2001) and in peanut the lipid concentration was far lower with addition of high amounts of NaCl (Hassanein 1999). Ion imbalance is also one of the factors contributing to the reduction of plant growth at higher salinity levels. Under high salinity conditions, Na^+ and Cl^- increase and substitute other ions (Ca^{2+}, K^+ and Mg^{2+}) (Khan 2001); this ionic imbalance causes an increase in the amino acid proline, which disturbs plant growth. Moreover, under higher salinity, reactive oxygen species accumulate (Bohnert & Jenson 1996). The rise of oxidative enzymes

reduces the metabolic activity in the plant and reduces growth (Rios-Gonzalez *et al*., 2002).

The results of the first experiment also showed a marked reduction in the final shoot biomass with increased salinity levels (Figure 4.3). The reason for the decreased shoot and leaf dry matter is likely to be the result of either salt toxicity or saline ions causing cell osmotic inequality (Abdel Latif 2010). Moreover, increasing salinity can decrease chlorophyll concentrations in the plant due to a deficiency in particular enzymes that produce photosynthetic pigments, leading to a reduction of carbohydrate supply and the size of the plant (Murkute *et al*., 2009).

Plant reproductive performance in the form of inflorescence number (which contained seeds) was slightly increased at 2.2 and 5 dS/m salinity (Figure 4.4). Inflorescence weight was noticeably increased in plants under low (2.2 dS/m) and medium (5 dS/m) salinity levels (Figure 4.5), whereas the high salinity level (10 dS/m) strongly diminished the inflorescence number and weight (Figures 4.4 and 4.5). Some plants can show an increase in growth parameters under certain levels of salinity. For example, the growth of *Salicornia rubra* Nels. increased under salinity stress, but at ~18 dS/m, the shoot biomass and other vegetation growth parameters decreased substantially (Khan 2001). Similar results were obtained for the legume *Alhagi pseudoalhagi* Bieb. where under medium levels (5 dS/m) of salinity the vegetative biomass increased, but decreased at 10 dS/m and higher levels of salt stress (Kurban *et al*., 1999).

Although mycorrhizal fungi have been found to survive and grow in saline conditions (Heofnagels *et al.,* 1993; Johnson-Green *et al.,* 1995), the mycorrhizas failed to colonise the plant roots under different salinity treatments as shown in the results. Some studies indicate that mycorrhizal growth can be reduced or that the fungi become unable to colonise the plant root under certain salinity stress conditions (Juniper & Abbott 1993). The same authors (2006) conducted an experiment on the effect of salinity addition on different species of mycorrhiza, and the results showed that spores of 11 species of fungi failed to germinate and form associations with the plant when salt was applied. Interestingly, the mycorrhizas in this experiment did not form an association with the roots even in the absence of the salt, indicating that salinity is not the only factor that can prevent the mycorrhizal spore germination. In fact, multiple factors can affect mycorrhizal spore germination making them unable to form associations with the plant root. For example, heavy metals in the soil can inhibit AM

fungi from making a successful root association (Bartolome-Esteban & Schenck 1994). Additionally, soil temperature can adversely affect spore germination (Tommerup 1983) and high levels of nutrients in the soil can reduce levels of mycorrhizal root colonisation (Bethlenfalvay 1992b). Thus, there are many factors in addition to salinity that can cause the failure of mycorrhizas to associate with the plant. For these reasons, it was not appropriate to interpret the data in the current experiments in the context of salinity and mycorrhizal interaction as the colonisation was, sadly, not clearly detectable and thus the results are discussed based only on the effect of different salinity levels on the plant to ensure accuracy of the interpretation.

4.4.2 Experiment 2: mixed salts

The investigation in the second experiment revealed important findings regarding the effect of salinity stress induced by various levels of mixed salts on *P. Lanceolata*. AM colonisation was also unsuccessful in this experiment even under the no-salt treatment. Thus, the AM fungi factor was eliminated from the discussion of plant ability to withstand salt stress, and I decided to concentrate only on the salt treatments and plant interaction. In this experiment, the effect of mixed salt salinity stress on different growth parameters followed a similar trend to the first experiment, and the effects of salinity were further extended to influence the offspring quality herein. Plant height and leaf number were markedly reduced, particularly at high salinity levels (Figures 4.8 and 4.9). It was previously reported that at 4 dS/m electrical conductivity, the soil is classified as saline and begins to affect plant growth (Brown 2008). Salinity stress was also shown to be associated with Cl^- accumulation in the leaves, causing injury and death of the foliage (Maas 1993). Experiments on sweet orange plants (*Citrus sinensis* L.) under salinity stress revealed a large reduction in leaf production and plant size with medium and high salt treatments (Syvertsen & Yelenosky 1988). Another experiment on carrizo citrange citrus (*C. sinensis* (L.) Osb. × *Poncirus trifoliata* L.) demonstrated striking damage due to salinity on growth parameters (Garcia-Sanches & Syvertsen 2006). The accumulation of high amounts of Na^+ and Cl^- in plant tissues during salinity stress is considered the major factor leading to the restriction of plant growth (Parida & Das 2005). According to this article, salinity is likely to affect plant growth by reducing the water potential and inducing ion disturbance, which leads to toxicity. *Plantago*

coronopus L. leaf osmotic potential was changed when exposed to salinity and reduced overall plant shoot growth (Koyro 2006). In barley plants, salinity stress was shown to cause ionic disturbance and growth suppression because of the accumulation of salt (Yang *et al*., 2009). In agreement with these data, the reduction of growth of both leaves and plant height in the current experiment was found at high salinity levels.

The reproductive performance was severely compromised under high salinity stress as manifested by the sharp decline in inflorescence number (Figure 4.10). Consistently, research has shown that high levels of salinity reduced the inflorescence production of the aquatic plants eurasian watermillfoil (*Myriophyllum spicatum* L.) and perfoliate pondweed (*Potamogeton perfoliatus* L.) (Twilley & Barko 1990). Even though these two species are considered to be ecologically adapted to saline habitats, when salinity increases above a certain level, the flowering is reduced. Another study on strawberry plants (Rapella cultivar) indicated a reduction in flower number due to the salinity of irrigation water (Awang & Atherton 1995). Gasim (1998) studied the effect of salinity stress on inflorescence growth in rape (*Brassica juncea* L.) in more detail. He showed an accumulation of the amino acid proline in flower tissues with increasing salinity stress with a concomitant reduction in chlorophyll concentration. The rise of proline was indicative of an osmotic stress that reduced the growth of the plant organs (Chandler & Thorpe 1987). Thereby, it is tempting to speculate that the osmotic stress and chlorophyll disturbance lead to the drop in inflorescence number of *Plantago* under high salinity stress.

Seed weight dropped substantially under salinity stress (Figure 4.11). Similar results were reported with peppergrass (*Lepidium latifolium* L.), in which the seed yield was reduced by 29% at the high salinity site (Leininger & Foin 2009). Another study on sunflower seeds produced under salinity stress also showed a decrease in seed yield above 4 dS/m, and the reduction of oil concentration inside the seeds was the main reason (Francois, 1996). A further experiment on chickpea (*Cicer arietinum* L.) showed a decline of seed production in plants exposed to high salinity stress due to increased Cl^- and reduced carbon content (Dua 1992).

Other parameters that were sharply reduced by high salinity levels were related to the plant offspring, the seed germination and the seedling length. A similar result was obtained with rabbit-foot grass (*Polypogon monspeliensis* L.), in which the seed quality and germination dropped drastically under high salinity (Callaway & Zedler 1998).

Consistent with these findings, exposure to salinity stress was shown to compromise the seed quality as manifested by the reduction of the seed coat and the induction of shape irregularities, in contrast to the seeds produced using good-quality water, which had a complete and smooth seed coat (Leininger & Foin 2009). Furthermore, seed examination revealed that salinity can cause seed dormancy, which reduces the quality and ability of seeds to germinate (Gulzar & Khan 2002). It was found that the growth-regulating hormones in the seed, such as ABA and others, inhibit seed production after exposure to salinity, and at the same time the hormone-induced seed germination is considerably decreased (Bewley & Black 1994). Accordingly, the results obtained in this investigation under high salinity levels agree well with the previous findings of the reduction of seed germination and quality of the seedlings in high salinity conditions.

The mycorrhizal inoculation failed to form an association with the plant at all levels of treatments. As mentioned above, there are many factors that can affect mycorrhizal spore germination and the successful association with the plant root. Different temperatures can influence spore germination and different mycorrhiza species have different temperature tolerances (Heinemeyer & Fitter 2003). Also, light has a major impact on mycorrhiza germination and the successful association with the plant (Aguirrezabal *et al*., 1994). It is tempting to speculate that the conditions under the controlled environment room where the experiments were conducted (especially the low light level) were not favourable to form a successful mycorrhizal association with the plant. Moreover, it is possible that the commercial mycorrhiza inoculum used went into a state of dormancy and the spores failed to germinate because of the unfavourable conditions (Tommerup 1983). It is therefore important for any future experiments to use different methods to obtain successful colonisations of mycorrhizas.

4.5 Conclusion

In both experiments, mycorrhizas failed to form a successful association with *P. lanceolata* plants. Thus, the experiments cannot confirm the negative or positive role of AM fungi in combating soil salinity or test the hypothesis. Overall, *Plantago* growth was relatively better under low and medium salinity levels, but at high salinity the growth could not be sustained. In the next set of experiments shown in Chapter 5, an expanded study of the AM fungal effects on the response to salinity stress was

conducted but under the real field conditions instead of the controlled environment state in order to overcome the limitations encountered in the present chapter. The experiments were also designed to include mixed salts, so as to mimic the field situation in a more realistic way, rather than the sole addition of pure NaCl. The findings of these experiments are demonstrated in the next chapter.

Chapter 5

The role of mycorrhizas in combating soil salinity under field conditions

5.1 Introduction

Plantago lanceolata, commonly known as ribwort plantain or English plantain, is a widely dispersed plant species found in various habitats (Van Tongeren & Van der Maarel 1985). The unique morphology and life history make this plant very adaptable to different ecological habitats (Van Groenendael & Slim 1988; Wolf 1988). *P. lanceolata* also acts as a weedy herb in areas disturbed by human activities (Tonsor *et al.*, 1993). In the natural environment, the plant mostly germinates during spring, and flowering, which commences in June and ends in mid-August, reaches the maximum during July (Tonsor 1987). *P. lanceolata* is wind-pollinated, with an average pollen-scattering distance of around 1.5 m (Tonsor 1985). Usually, the seeds mature in late summer and are mostly dispersed during autumn (Bos *et al.* 1986). After maturation, the seeds can remain in the soil, forming a seed bank (Cavers *et al.* 1980). The plant can form successful associations with a wide variety of arbuscular mycorrhizal (AM) fungal species (Smith & Read 1997). The root analysis of *P. lanceolata* revealed its ability to form associations with major genera of mycorrhizas, namely *Glomus*, *Acaulospora*, *Entrophospora*, *Gigaspora*, and *Scutellospora* (Klironomos & Hart 2002). Oehl *et al.* (2004) used *Plantago* plants as trap cultures for field study and found approximately 35 different AM species associated with the roots. The mycorrhizal associations have differential impacts on *P. lanceolata* growth and behaviour. In this regard, Bever (2002) showed that maximum growth benefits occurred after *P. lanceolata* colonisation by *Acaulospora morrowaiae* and *Archaespora trappei* but not *Scutellospora calospora*. In another study by Bennett and Bever (2007) assessing the impacts of three mycorrhiza species on *P. lanceolata* growth and herbivore resistance, growth promotion was found to be optimal with *Glomus white* mycorrhizas, modest with *Archaespora trappei*, and completely absent with *S. calospora*. Mycorrhizal fungal colonisation can give contradictory results when grown under controlled conditions (glasshouse) or in field situations. Some glasshouse studies indicated that high levels of phosphorus in the soil reduced the mycorrhizal root association (Mendoza & Pagani 1997; Cornwell *et al.*,

2001). In open grassland, Sanders and Fitter (1992) found no relation between increasing phosphorus and other nutrient elements with decreasing or increasing mycorrhizal colonisation of the roots. Another experiment under grassland field conditions showed that mycorrhizal associations increased only in certain seasons of the year, especially during the growing season, despite the high phosphorus levels in the soil (Garcia & Mendoza 2007).

There are many factors that render mycorrhizal effects under field conditions different from those in controlled environments. Under field conditions, soil organisms like earthworms, arthropods, and nematodes can either enhance mycorrhizal effects by dispersing spores or reduce the mycorrhizal benefits by eating hyphae and using them as a food source (Gange 1993; Johnson *et al.*, 2005; Sjursen *et al.*, 2005). In addition, the distribution and function of different mycorrhizal species in field situations are controlled by multiple factors, such as soil pH, nutrient amount, and salinity (Abbott & Robinson 1991). The variation in field vegetation cover determines the existence and behaviour of different mycorrhizal species, as well. As such, *Glomus aggregatum* and *G. leptotichum* were common in a range of sites of different vegetation cover, but *G. occultum* and *G. macrocarpum* appeared only with certain kinds of plant species (Johnson *et al.*, 1992). The differences in soil moisture and hydrologic conditions also affect various mycorrhizal species under field conditions; for example, *Glomus fasciculatum* and *G. intraradices* were abundant during the dry season and decreased substantially during the rainy season (Escudero & Mendoza 2005).

Different species of mycorrhizas have distinct abilities to influence plant growth (Daei *et al.*, 2009). Hence, it has been recommended to use multiple combinations of mycorrhizal species rather than a single species to optimise the beneficial effects on plant growth (Koide 2000; Alkan *et al.*, 2006). Combined inoculation with *G. fasciculatum* and *G. macrocarpum* mycorrhizas has been shown to be superior to single inoculation in enhancing *Acacia* plant growth under salinity stress (Giri *et al.*, 2003). The positive effect of using combined species of mycorrhizas can be attributed to the synergistic interaction between the fungal species, adding more benefits to the plant (Sharma *et al.*, 1996). Moreover, different species of mycorrhizas can form large underground mycelial networks that can increase the absorption of water and nutrients by the plant (Colla *et al.*, 2008; Daei *et al.*, 2009). However, there is no general consensus among researchers that the beneficial effects of mixed mycorrhizas are common to all situations. For instance, the single inoculation of *Astragalus* plants with

G. intraradices yielded more shoot dry matter biomass than a combination of *G. mosseae*, *G. claroideum*, and *G. intraradices* under salinity stress (Peng *et al.*, 2011). In some situations, using multiple or even individual species of mycorrhizas can result in parasitic effects on the plant by draining resources from the roots, resulting in suppression of growth (Johnson *et al.*, 1997).

Very few studies have been conducted on the effect of mycorrhizas on seed production and second-generation seedling growth patterns (Koide 2010). It has been reported that mycorrhizas can enhance seed production and elevate their quality in an indirect manner through the reduction of herbivore and disease attack (Hendrix 1988; Lee 1988). It has also been suggested that mycorrhizas can affect seed production and quality by enhancing nutrient absorption, particularly phosphorus (Stanley *et al.*, 1993; Koide *et al.*, 1994). Yet, different mycorrhizal species, such as *Vigana unguiculata*, can have different influences on seed yield (Muthukumar & Udaiyan 2002). In the latter experiment, *Scutellospora calospora* and *Glomus aggregatum* were found to enhance the seed yield more than other mycorrhizal species. In another experiment on soybean (*Glycine max* L, Merr), the addition of different species of mycorrhizas (*Glomus mosseae, G. etunicatum*, and *Gigaspora rosea*) resulted in variable amounts of phosphorus and lipid in the seeds (Bethlenfalvay *et al.*, 1997). Thus, it is important to test the effects of different mycorrhizal species and their behaviour in the context of seed germination and second-generation seedling growth.

The goal of the current study was to examine the effect of using multiple or single species of mycorrhizas on the performance of *P. lanceolata* under field conditions during the exposure to salinity stress. It was hypothesised that using multiple species of fungi under field and salinity conditions would result in enhanced resistance of parental plants to salt stress and better growth of the offspring compared to the use of single species of mycorrhizas.

5.2 Materials and methods

Plantago lanceolata (section 2.6.1) seeds were germinated in a controlled environment room (section 2.1). When the germinated seedlings reached the four-leaf stage, they were transplanted into 11-cm square pots filled with commercial sterilised compost (section 2.1.3). During the process of potting the plants, half of the seedlings were

treated with mycorrhizas, and the other half were kept without treatment as controls. The potted plants were transferred and kept in the glasshouse at a temperature range of 20–28°C in daylight supplemented overnight with high-pressure sodium vapour lamps and a relative humidity between 50–85%. The plants were maintained in the glasshouse for approximately two weeks to establish the mycorrhizal-plant association and watered daily as required before transfer to the open-field conditions.

The field study and plant transfer took place during a British summer season between the beginning of May and the end of August. A field plot measuring 12 m x 9 m was selected and fenced with metal mesh supported with rods. For the first experiment, a metal mesh 30 cm inside the ground and 1 m high was installed, but for the second field experiment a mesh 30 cm underground and 2.5 m high was fitted. The metal mesh fencing was used as a repellent for mammalian herbivores, especially rabbits and deer. Inside the field plot, the plants were arranged in a complete randomised block design, with a space of 50 cm between each plant.
The overall study was divided into two different experiments:

- *Commercial mycorrhizas experiment (First experiment)*

A commercial mix of mycorrhizas (section 2.2.1) was used in this experiment. Treatment conditions were with or without mycorrhizas, four levels of salinity, and two types of salts (NaCl and mixed) with six replicate plants, which gave a 2 x 4 x 2 x 6 factorial and a total of 96 plants.

- *Individual species of mycorrhizas experiment (Second experiment)*

The two types of mycorrhizal species used in this experiment were *Glomus mosseae* and *G. etunicatum*, which were inoculated singly and in combination with an untreated control species. Only mixed salts were used (with and without), with three levels of salinity. The experiment used six replicates of plants, which gave a 2 x 3 x 2 x 6 factorial and a total of 72 plants.

In both field experiments (first and second experiments), mixed salts (see Appendix) were used as the experimental treatment. The first field experiment (using

commercial mycorrhizas) used different salt types (NaCl and mixed) and four salinity levels of electrical conductivity (2.2, 5, and 10 dS/m at 25°C and a control). The second experiment used only mixed salts without NaCl to mimic the real field situation with three salinity levels of electrical conductivity (1.5 and 3.5 dS/m at 25°C and a control). Every week, 200 mL of salt solution was added to each desired plant treatment to prevent the salinity level in the soil from flushing away.

Both experiments were grown for four months, after which plants were harvested and different plant parameters were recorded, including height, leaf number, inflorescence number, and length. The weight of inflorescences was taken to indicate the weight of the seeds contained. The harvested shoots of plant weight were taken and considered as initial shoot biomass as well as final dry shoot weight (section 2.4). Roots for each plant were cleaned and stained for mycorrhizal visualisation (section 2.5.1). The stained roots were prepared on glass slides for AM fungal quantification and identification of different parts of mycorrhizas such as vesicles, hyphae, and arbuscules (section 2.5.2).

The seeds produced from F1 plants treated under the salinity conditions explained above were used for the germination of F2 generation plants. From each plant, 15 healthy seeds were selected for the germination test. Petri dishes of 90-mm diameter with filter paper inside were used for the germination test under constant room temperature (26°C). The seeds of each plant were divided into three Petri dishes, and five seeds were placed in each Petri dish for a total of 15 seeds for each plant. The seeds were watered daily with distilled water, and daily seedling germination was recorded for seven days of the experiment. Final total shoot and root lengths for each successfully germinated seedling were recorded.

Data were tested for normality and then analysed by Analysis of Variance (ANOVA) using the Unistat (version 6.0) statistical package. Tukey's test was used to separate the means of treatments involved. Statistical tests employed salt type, salt level, and mycorrhizas as main effects.

5.3 Results of the first field experiment

5.3.1 First generation

There was a significant difference in the effect of salt type on mean leaf number (Table 5.1), with NaCl tending to produce plants with higher number of leaves than those treated with mixed salts (Figure 5.1). Overall, the mycorrhizal-association factor alone did not have any effect on mean leaf number (Table 5.1). However, the addition of mycorrhizas enhanced mean leaf number at the 2.2 dS/m salinity level only, leading to a significant interaction term between salinity and AM (Table 5.1; Figure 5.1). With respect to mean final plant height, no effect of salt or mycorrhizal inoculation was found (Table 5.1; Figure 5.2). The mean dry shoot biomass showed a significant effect of salt type only (Table 5.1), as the addition of mixed salts reduced shoot biomass more than the addition of NaCl alone (Figure 5.3).

Table 5.1: Summary of the results of Analysis of Variance of different parameters in different types of treatments. Salt types (NaCl and mixed salts), salinity levels (EC) (0, 2.2, 5, and 10 dS/m), and mycorrhizal treatment (AM). Degrees of freedom for salt types = 1, 45; salinity levels = 2, 45; AM = 1. 45.

	Leaf number		**Plant height (cm)**		**Shoot dry biomass (g)**	
	F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
Salt type	3.9	< 0.05	1.8	0.2	4.7	< 0.05
Salinity (EC)	0.3	0.74	1.2	0.3	1.3	0.3
AM	0.4	0.5	1.2	0.3	0.04	0.8
Salt type x salinity (EC)	1.8	0.2	0.7	0.5	2.6	0.08
Salt type x AM	1.2	0.3	0.2	0.7	0.4	0.5
Salinity (EC) x AM	3.8	< 0.05	1.1	0.3	2.0	0.1
Salt type x salinity (EC) x AM	0.95	0.4	1.0	0.4	1.8	0.17

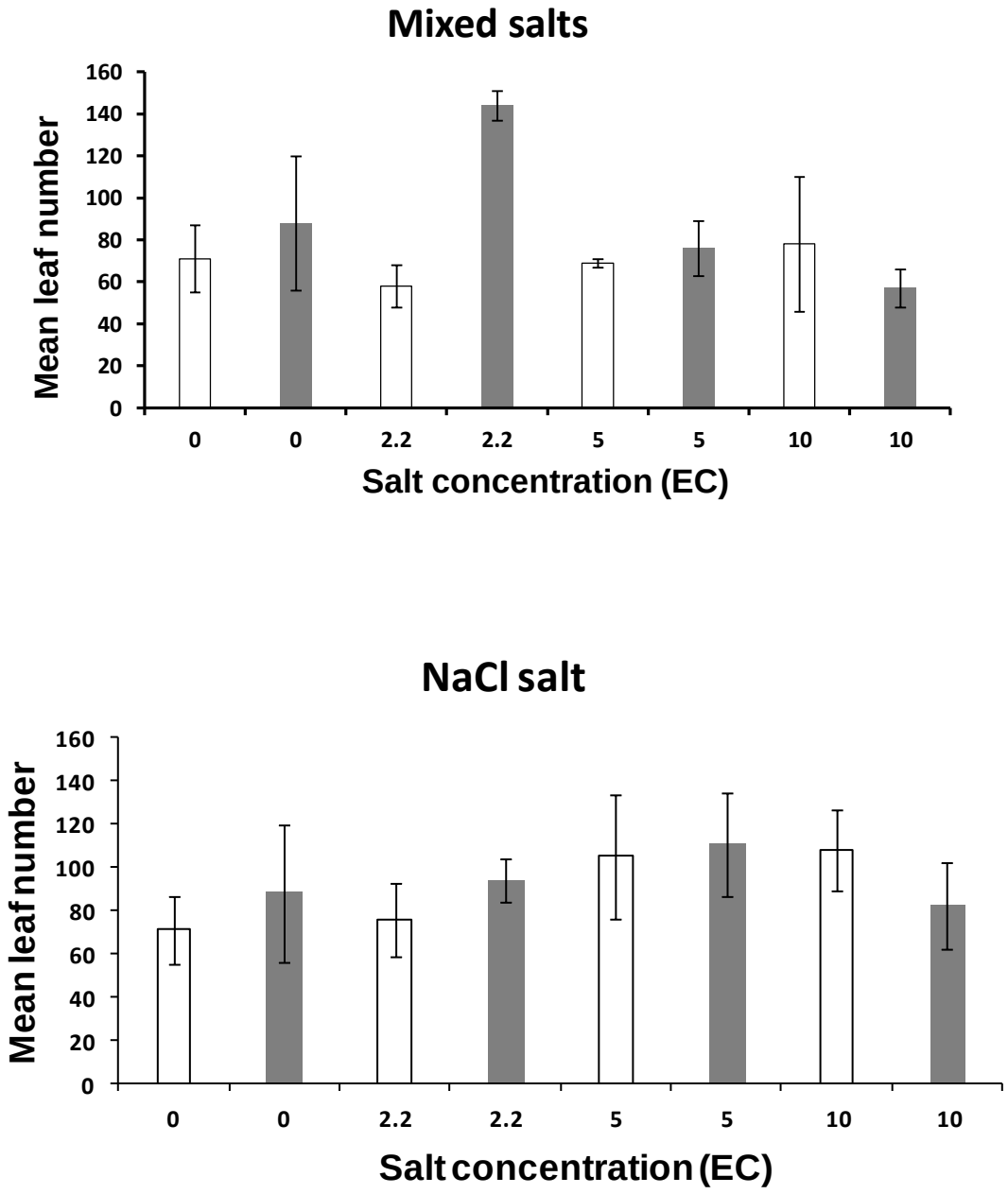

Figure 5.1: The final leaf plant count for different salinity types (mixed and NaCl) and different salinity levels (EC) (0, 2.2, 5, and 10 dS/m), with (grey bars) and without (white bars) commercial mycorrhizal inoculation.

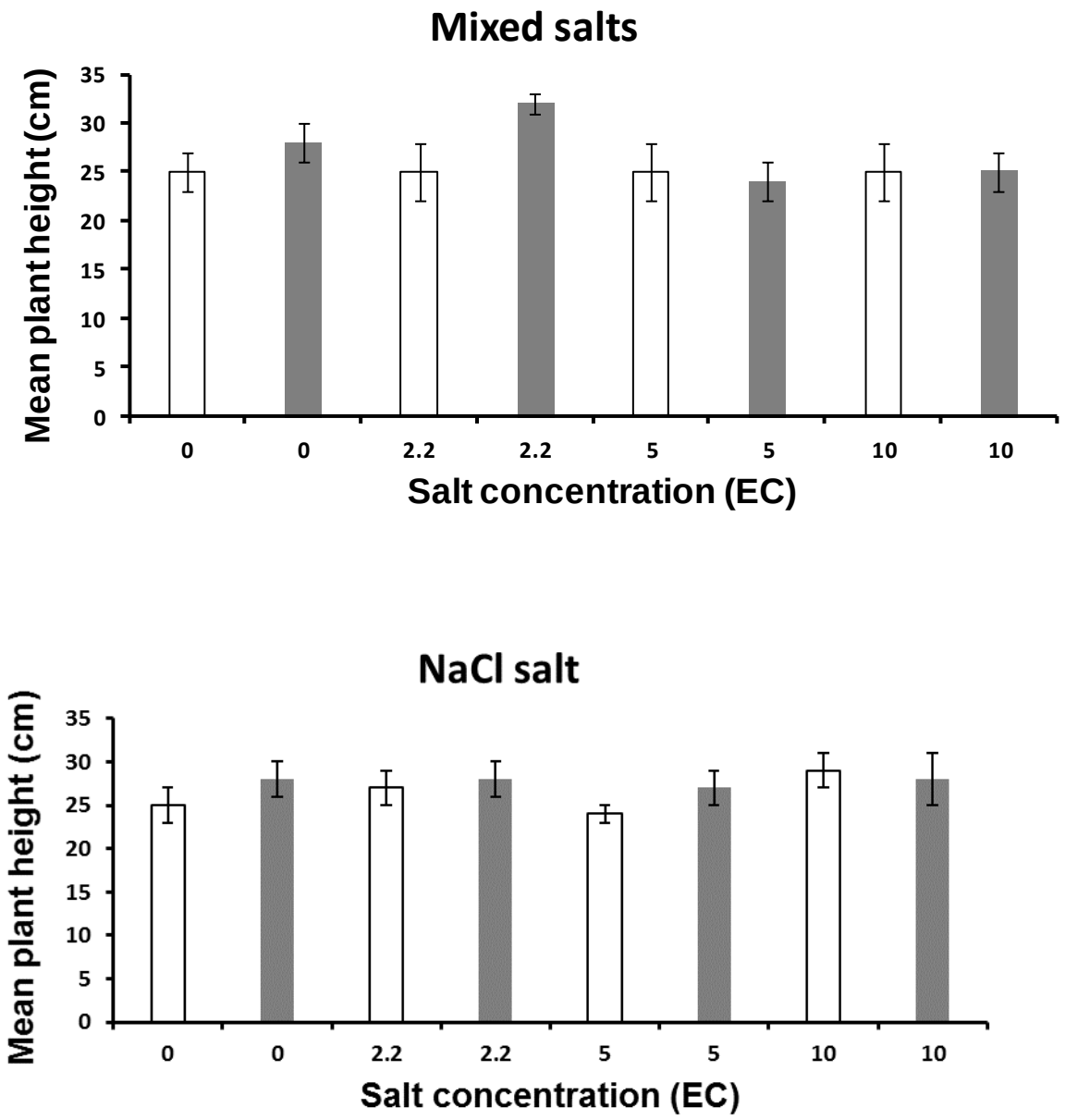

Figure 5.2: The final plant height in cm for different salinity types (mixed and NaCl) and different salinity levels (EC) (0, 2.2, 5, and 10 dS/m), with (grey bars) and without (white bars) commercial mycorrhizal inoculation.

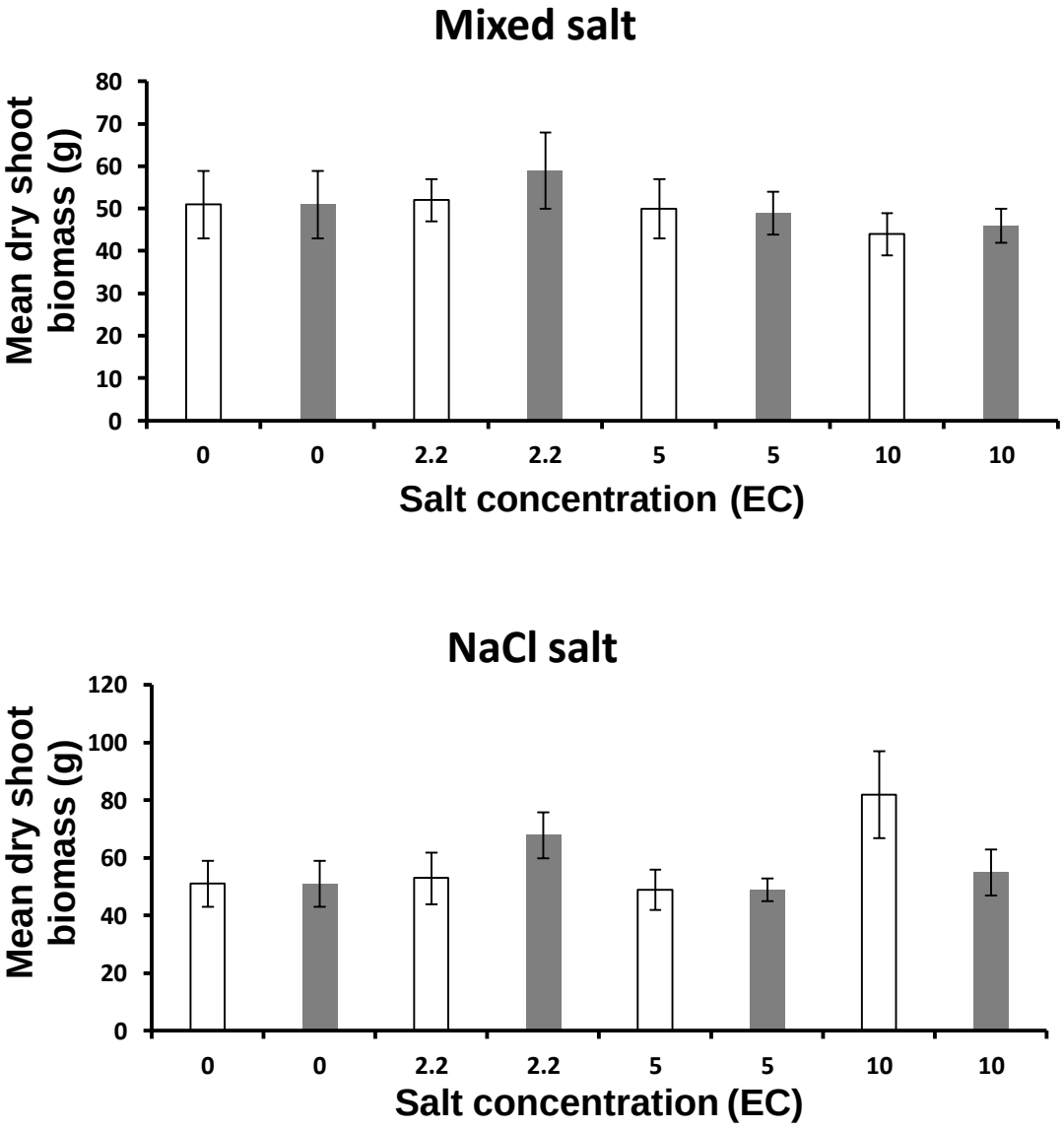

Figure 5.3: The final plant shoot biomass after oven-drying for different salinity types (mixed and NaCl) and different salinity levels (EC) (0, 2.2, 5, and 10 dS/m), with (grey bars) and without (white bars) commercial mycorrhizal inoculation.

Production of inflorescences was affected by the type of salt addition (Table 5.2). Addition of NaCl salt enhanced the mean inflorescence number more than the non-salt situation or the addition of mixed salts (Figure 5.4). Mycorrhizal addition had no overall effect on mean inflorescence number, but a significant interaction was found between fungal addition and salinity levels (Table 5.2). This was because at low salinity stress (2.2 dS/m) mycorrhizas enhanced inflorescence number, but with increasing salinity stress levels this positive effect disappeared (Figure 5.4). On the other hand, the mean inflorescence length (cm) was not affected by any of the treatments added to the

experiment (Table 5.2). The mean seed weight was reduced by the addition of different salt types (Table 5.2). Using mixed salts decreased the weight of seeds produced by the plant far more than the addition of NaCl salt (Figure 5.5). Regarding the effect of different salinity levels (Table 5.2), medium saline addition produced larger seeds of greater weight (Figure 5.5). The medium salinity level produced higher seed weight in comparison with lower salinity addition and no salt treatment (Figure 5.5). The addition of mycorrhizas did not show any positive effect on mean seed weight produced by the plants under different salinity effect factors (Table 5.2).

Table 5.2: Summary of the results of Analysis of Variance of different parameters for different types of treatments. Salt types (NaCl and mixed salts), salinity levels (EC) (0, 2.2, 5, and 10 dS/m), and mycorrhizal treatment (AM). Degrees of freedom for salt types = 1, 45; salinity levels = 2, 45; AM = 1, 45.

	Inflorescence number		**Inflorescence head length (cm)**		**Seed weight (g)**	
	F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
Salt type	7.0	< 0.01	0.4	0.5	6.3	< 0.01
Salinity (EC)	0.6	0.5	0.2	0.8	2.9	< 0.05
AM	1.8	0.2	0.1	0.8	0.01	0.9
Salt type x salinity (EC)	1.0	0.4	0.09	0.9	0.06	0.9
Salt type x AM	1.7	0.2	2.3	0.1	1.4	0.2
Salinity (EC) x AM	6.3	< 0.001	0.6	0.5	1.1	0.3
Salt type x salinity (EC) x AM	0.54	0.59	1.4	0.3	0.7	0.5

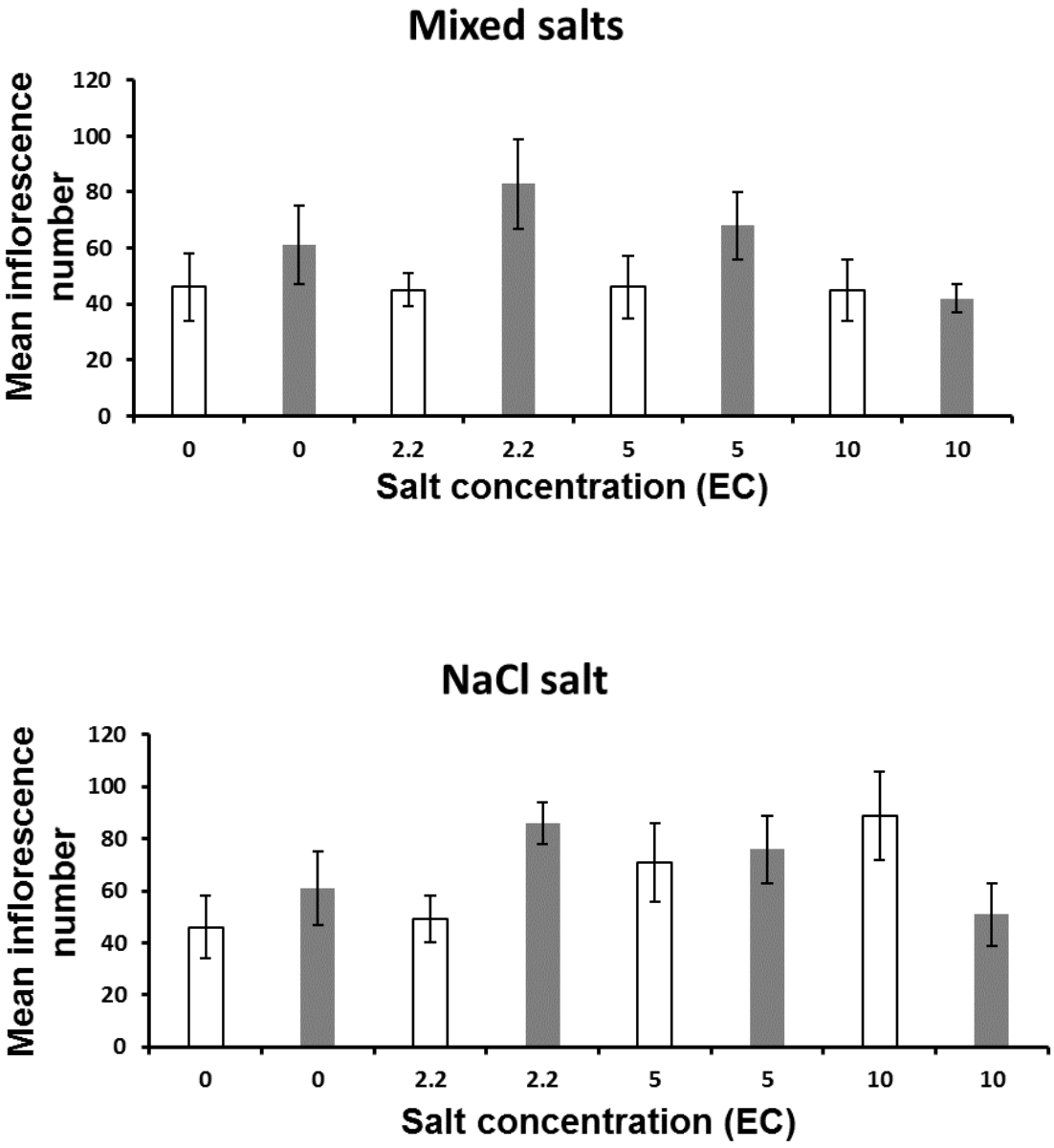

Figure 5.4: The inflorescence number for different salinity types (mixed and NaCl) and different salinity levels (EC) (0, 2.2, 5, and 10 dS/m), with (grey bars) and without (white bars) commercial mycorrhizal inoculation.

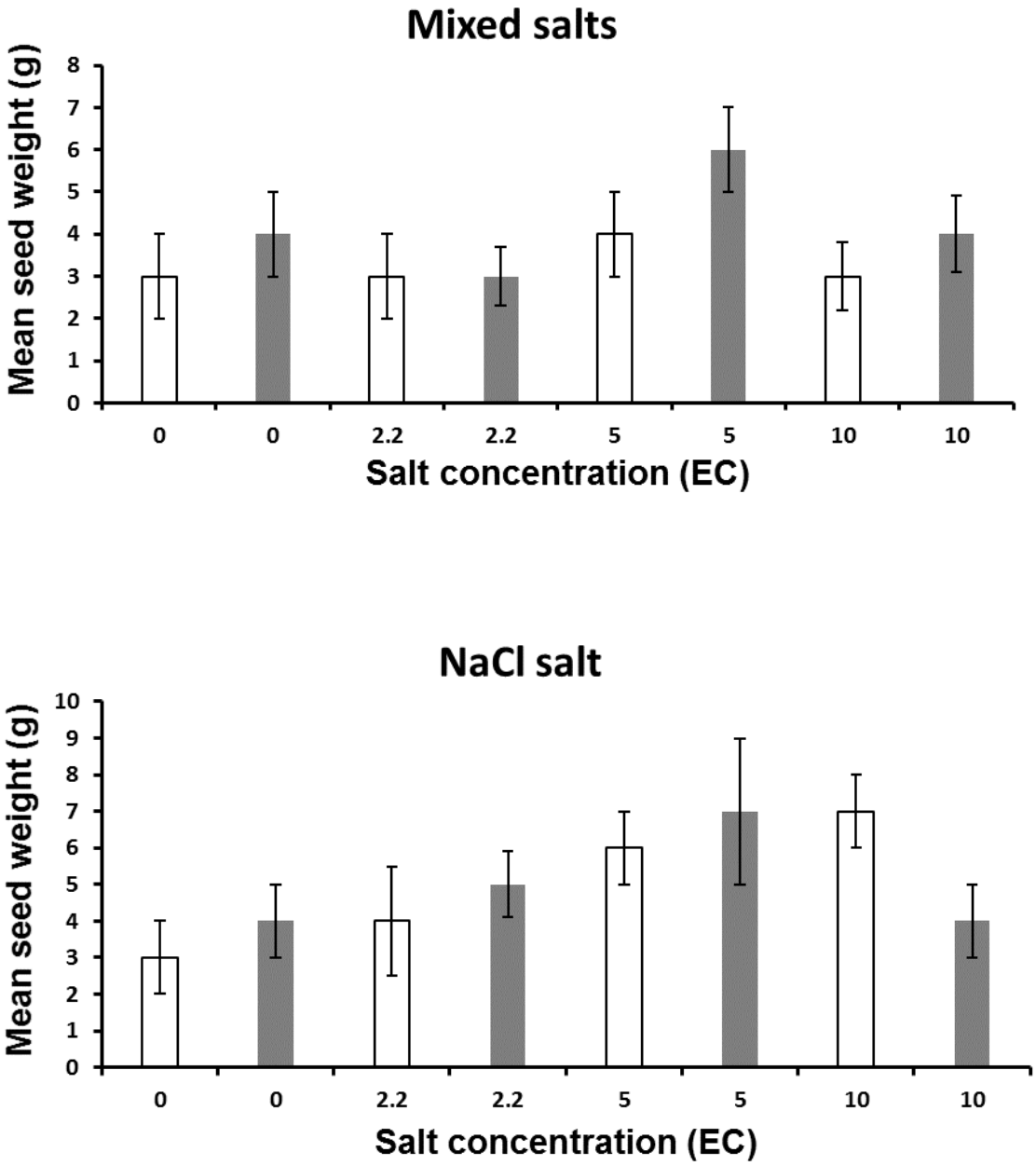

Figure 5.5: Seed weight production in grams for different salinity types (mixed and NaCl) and different salinity levels (EC) (0, 2.2, 5, and 10 dS/m), with (grey bars) and without (white bars) commercial mycorrhizal inoculation.

5.3.2 Mycorrhizal root colonisation

Mycorrhizal colonisation was successfully detected in the roots with the average percentage values summarised in Table 5.4. Hyphal root colonisation was significantly influenced by the interaction between salt type and salinity, showing different patterns of colonisation across different salinity types and different levels of salinity stress (Table 5.3). The medium salinity stress (5 dS/m) with mixed salts reduced the incidence of hyphal colonisation, but with NaCl salt, the hyphal colonisation increased across the other treatments (Table 5.4). The other mycorrhizal parts (vesicles and arbuscules) did not show any significant differences across treatments (Table 5.3). On the other hand, there was a significant interaction between the spore incidence and salinity levels (Table 5.5), as spores appeared at higher salinity levels only (Figure 5.6). With increasing salinity levels, the number of spores produced by mycorrhizas increased, especially at 5 and 10 dS/m (Figure 5.6). At lower salinity levels (0 and 2.2 dS/m), however, mycorrhizas did not produce spores during their association with plant roots (Figure 5.6). There was also a significant interaction term between salt type and salinity level (Table 5.5). This was because the addition of mixed salts produced spores at both medium and high salinity levels, but with NaCl salt spores were only seen at the high level of salinity (10 dS/m) (Figure 5.6).

Table 5.3: Summary of the results of Analysis of Variance of different root colonisation by mycorrhizas for different types of treatments. Salt types (NaCl and mixed salts), salinity levels (EC) (0, 2.2, 5, and 10 dS/m), and mycorrhizal treatment (AM). Degrees of freedom for salt types = 1, 58; salinity levels = 2, 58; AM = 1, 58.

	Hyphae		**Vesicles**		**Arbuscules**	
	F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
Salt type	0.8	0.4	0.4	0.5	0.13	0.7
Salinity (EC)	0.1	0.9	0.3	0.7	0.2	0.8
AM	0.3	0.6	0.02	0.9	0.01	0.9
Salt type x salinity (EC)	3.0	< 0.05	1.5	0.2	1.3	0.3
Salt type x AM	2.0	0.2	1.9	0.2	0.1	0.8
Salinity (EC) x AM	0.1	0.9	1.8	0.2	1.2	0.3
Salt type x salinity (EC) x AM	1.1	0.3	1.1	0.3	0.5	0.6

Table 5.4: Mean mycorrhizal colonisation percentages (%) for different treatments after root-staining method. H = hyphae; V = vesicles; A = arbuscules.

Salt types	Mycorrhizas	Salinity level	H %	V %	A %
Mixed	AM	2.2	39 ± 7	6 ± 3.7	5.6 ± 2.3
		5	37 ± 3.5	7 ± 4.7	1.2 ± 0.7
		10	36 ± 8.7	16 ± 8.5	3.4 ± 2.9
	No	2.2	50 ± 5	23 ± 6.9	3 ± 1.4
		5	32 ± 4.7	6 ± 2.4	3 ± 1.1
		10	48 ± 6.7	14 ± 6.6	3 ± 2.4
NaCl	AM	2.2	39 ± 6.9	10.6 ± 4.3	1 ± 0.4
		5	44 ± 3.1	11.5 ± 6.1	1.2 ± 0.5
		10	42 ± 7	16 ± 6.7	4 ± 2.6
	No	2.2	29 ± 8.5	9 ± 6.3	1 ± 0.5
		5	43 ± 6.3	12 ± 4	5 ± 3.7
		10	32 ± 7.3	3 ± 0.9	2.3 ± 0.9
No Salt	AM	0	41 ± 7	14 ± 3.9	1.5 ± 0.6
	No	0	47 ± 7	11 ± 2.9	6.8 ± 5.8

Table 5.5: Summary of the results of Analysis of Variance of mycorrhizal spore production obtained under different field experiment conditions. Degrees of freedom for salt types = 1, 60; salinity levels (EC) = 2, 60; AM = 1, 60.

	Spores produced	
	F-value	**P-value**
Salt type	0.1	0.7
Salinity (EC)	3.9	< 0.05
AM	0.14	0.7
Salt type x salinity (EC)	5.0	< 0.01
Salt type x AM	1.3	0.3
Salinity (EC) x AM	0.1	0.9
Salt type x salinity (EC) x AM	0.4	0.7

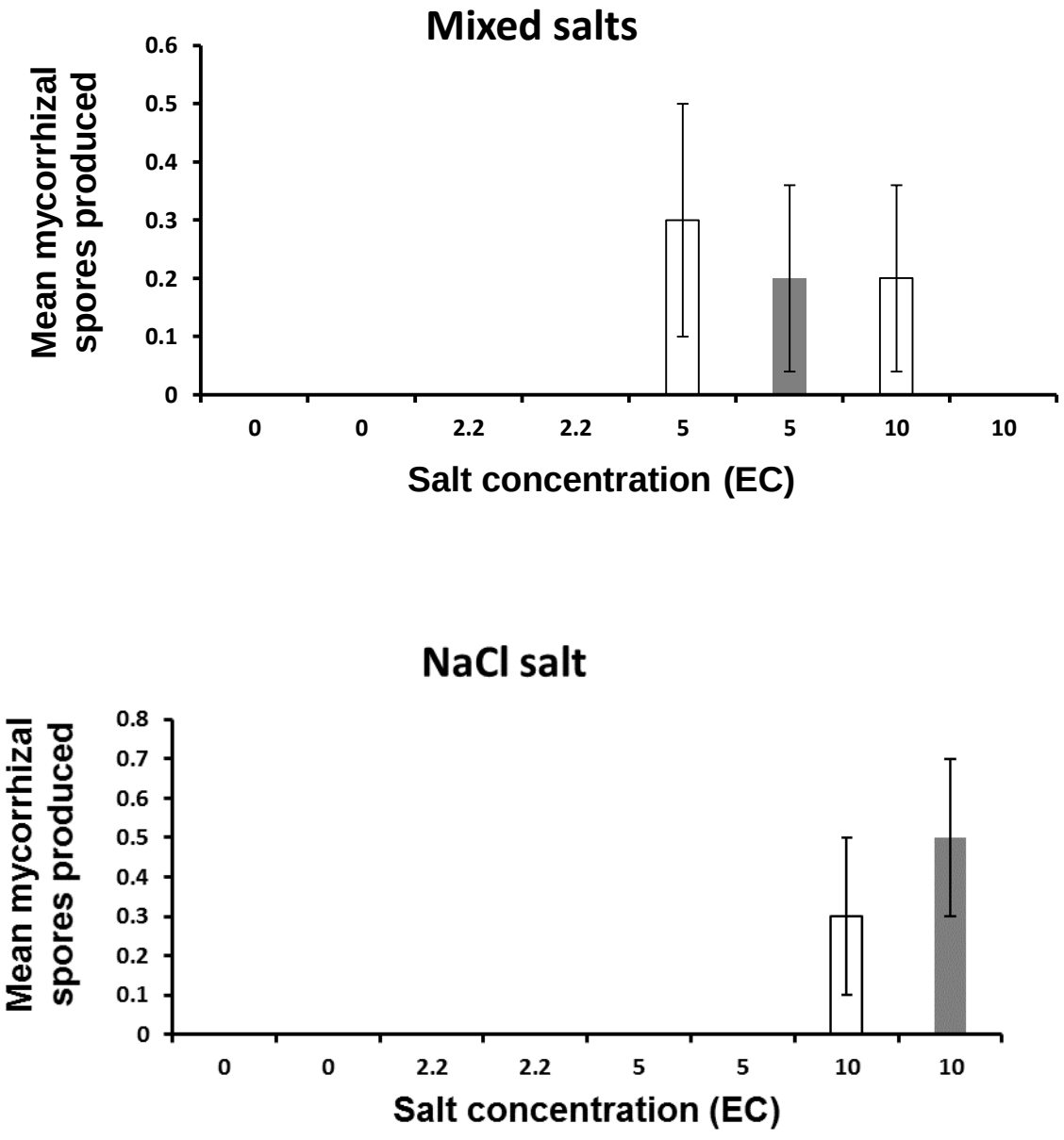

Figure 5.6: Number of mycorrhizal spores observed for different salinity types (mixed and NaCl) and different salinity levels (EC) (0, 2.2, 5, and 10 dS/m), with (grey bars) and without (white bars) commercial mycorrhizal inoculation.

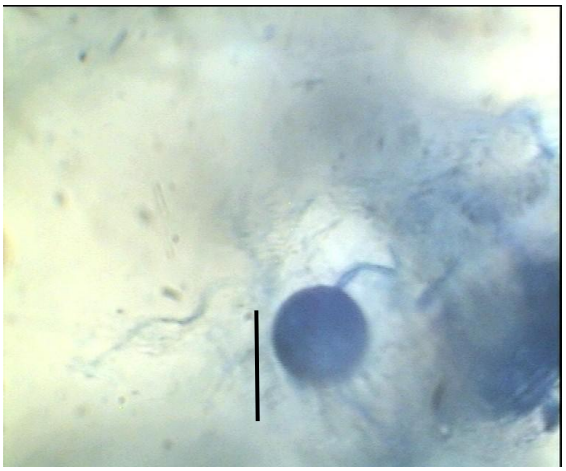

Figure 5.7: An example of a mycorrhizal spore produced during association with plant root under salinity stress (scale bar 100µm). At medium and high salinity stress, mycorrhizas tend to produce more spores, but at the control and low salinity stress, mycorrhizas did not form spores.

5.3.3 Second generation

Neither salt types nor different levels of salinity gradients had any effect on second-generation mean-seedling length (Table 5.6). Only the addition of mycorrhizas in the field to parental plants produced a significant result and increased seed germination (Table 5.6; Figure 5.8). The addition of mycorrhizas enhanced the mean seed germination of the offspring more than non-inoculated parental plants with mycorrhizas with no salt addition, but with salinity addition, the mycorrhizal addition did not show any enhancement (Figure 5.8).

Table 5.6: Summary of the results of Analysis of Variance for second-generation seed germination and seedling length. Salt types (NaCl and mixed salts), salinity levels (EC) (0, 2.2, 5, and 10 dS/m), and mycorrhizal treatment (AM). Degrees of freedom for salt types = 1, 60; salinity levels = 2, 60; AM = 1, 60.

	Seed germination		**Seedling length (cm)**	
	F-value	**P-value**	**F-value**	**P-value**
Salt type	0.2	0.7	2.6	0.1
Salinity (EC)	0.6	0.6	0.8	0.4
AM	5.4	< 0.05	1.6	0.2
Salt type x salinity (EC)	0.6	0.5	0.1	0.9
Salt type x AM	0.2	0.7	0.1	0.7
Salinity (EC) x AM	0.8	0.4	1.3	0.3
Salt type x salinity (EC) x AM	1.6	0.2	2.6	0.08

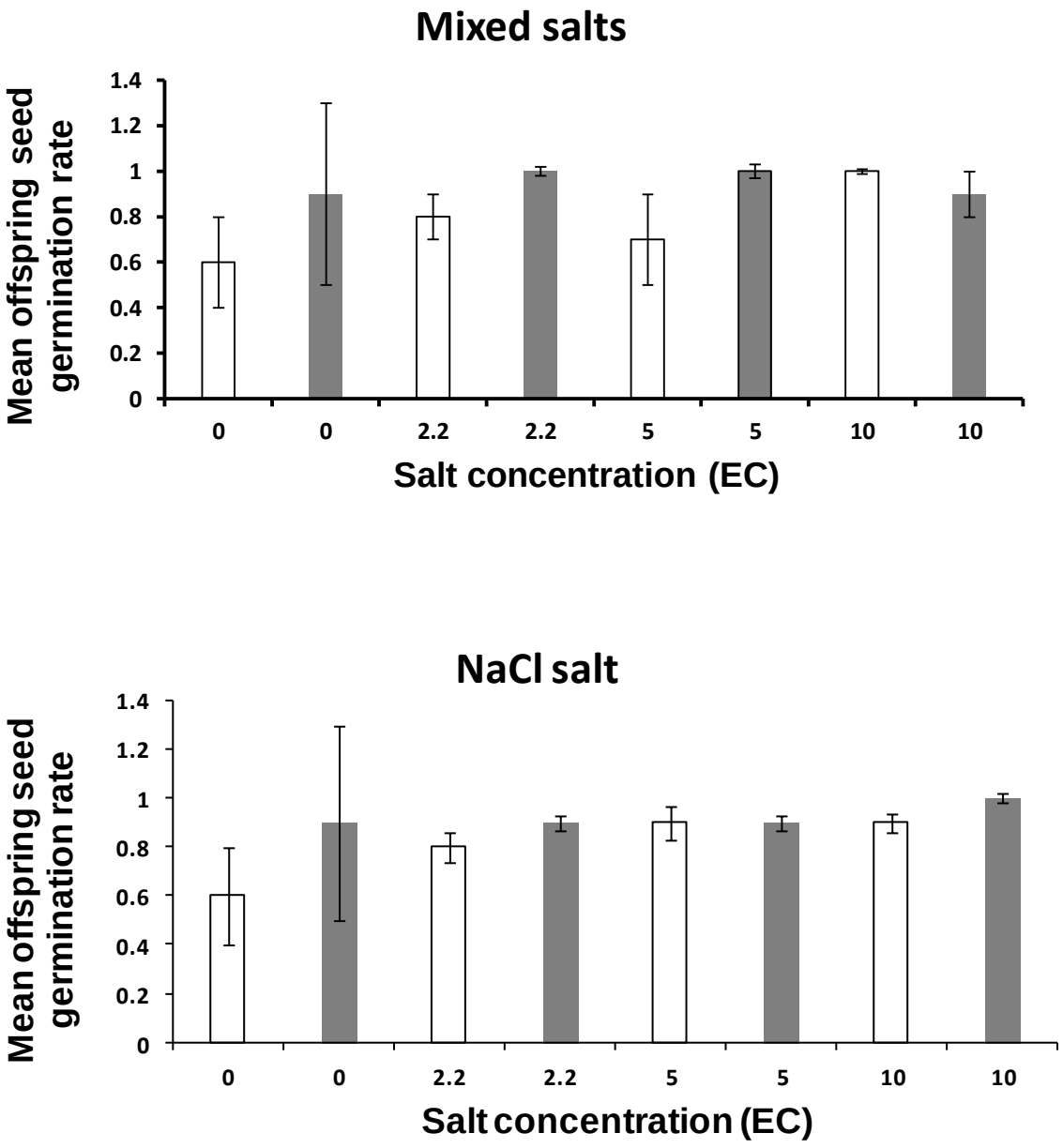

Figure 5.8: Seed germination rate of second-generation plants under lab conditions and their parental mycorrhizal association effect in field conditions for different salinity types (mixed and NaCl) and different salinity levels (EC) (0, 2.2, 5, and 10 dS/m). Groups shown are with (grey bars) and without (white bars) commercial mycorrhizal inoculation.

5.4 Results of the second field experiment

5.4.1 First generation

In this set of experiments, there were very few significant effects of different salinity levels or mycorrhizal species on different plant vegetative or reproductive parameters (Table 5.7; Table 5.8). Even in flowering stages and seed production, mycorrhizal addition had no effect in the field (Table 5.8). The one exception was seen with mean plant height (Table 5.7). Plants inoculated with *Glomus etunicatum* were taller than those treated with *G. mosseae* mycorrhizal fungi (Figure 5.9).

Table 5.7: Summary of the results of Analysis of Variance for different salinity levels (EC) (0, 1.5, and 3.5 dS/m) and AM treatment (Gm = *Glomus mosseae*, Ge = *G. etunicatum*, and the two combined). Degrees of freedom of salinity levels = 2, 56; Gm = 1, 56; Ge = 1, 56.

	Leaf number		Plant height (cm)		Shoot dry biomass (g)	
	F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
Salinity (EC)	0.72	0.49	0.89	0.43	0.79	0.46
Gm	0.83	0.37	2.6	0.11	1.9	0.18
Ge	0.2	0.66	5.2	< 0.05	2.4	0.12
Salinity (EC) x Gm	0.42	0.66	0.18	0.84	0.36	0.7
Salinity (EC) x Ge	0.54	0.59	1.1	0.34	3.0	0.07
Gm x Ge	0.25	0.62	0.88	0.35	0.07	0.79
Salinity (EC) x Gm x Ge	0.19	0.82	0.1	0.94	0.55	0.58

Table 5.8: Summary of the results of Analysis of Variance for different plant parameters at different salinity levels (EC) (0, 1.5, and 3.5 dS/m) and AM treatment (Gm = *Glomus mosseae*, Ge = *G. etunicatum*, and the two combined). Degrees of freedom of salinity levels = 2, 56; Gm = 1, 56; Ge = 1, 56.

	Inflorescence number		Seed weight (g)	
	F-value	P-value	F-value	P-value
Salinity (EC)	0.24	0.78	1.1	0.33
Gm	0.63	0.43	0.43	0.51
Ge	1.1	0.3	0.91	0.35
Salinity (EC) x Gm	0.61	0.55	1.2	0.3
Salinity (EC) x Ge	1.4	0.26	1.3	0.32
Gm x Ge	0.67	0.41	0.11	0.74
Salinity (EC) x Gm x Ge	1.7	0.19	0.02	0.98

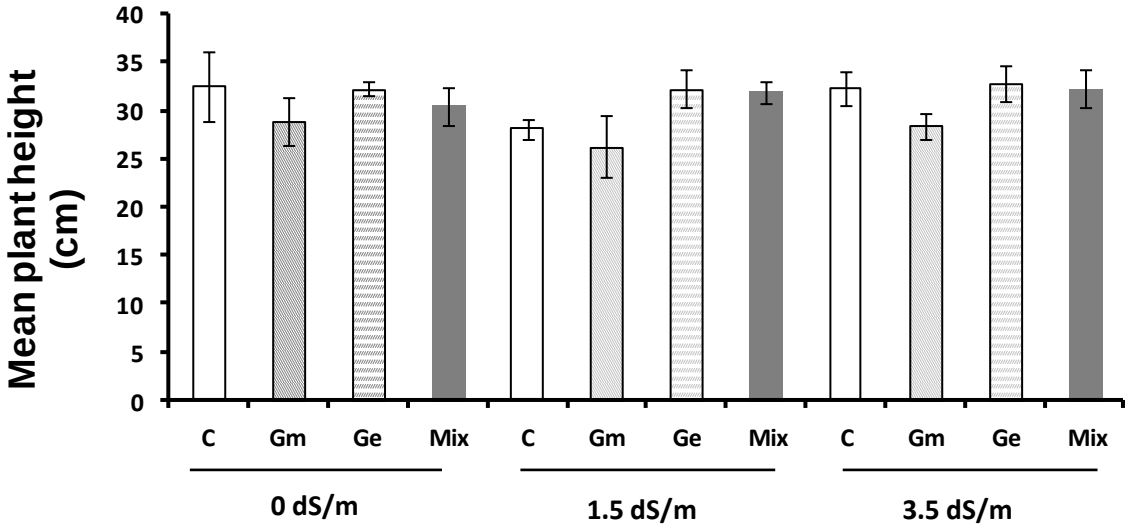

Figure 5.9: Plant height (cm) for salinity treatments with different species of mycorrhizas. Ge, *G. etuicatum*; Gm, *G. mosseae*; Mix, adding *G. etunicatum* + *G. mosseae*; C, control. Salt salinity levels (0, 1.5, and 3.5 dS/m).

5.4.2 Mycorrhizal root colonisation

The addition of mycorrhizal species was associated with different colonisation rates of hyphae under salt stress (Table 5.9). Addition of species of mycorrhizas had no effect on hyphal root colonisation at the low salinity level (1.5 dS/m), but at no salt addition (0 dS/m) and high salinity (3.5 dS/m) stress, the different species of mycorrhizas produced a remarkable increase in hyphal colonisation, more than non-inoculated plants (Figure 5.10).

Overall, the addition of salt had no effect on vesicle colonisation rate (Table 5.9). However, a significant interaction term was found between salinity and the AM species (Table 5.9; Figure 5.11). At no salt stress, *G. mosseae* and *G. etunicatum* increased the production of vesicles, but this effect was lost with salinity addition in comparison with non-inoculated plants (Figure 5.11)

Arbuscules were not affected by salinity stress nor the addition of different mycorrhizas species; yet, the interaction of these factors resulted in a significant influence on arbuscule production (Table 5.9). Inoculation with *G. mosseae* and *G. etunicatum* had no effect on arbuscule formation at low (1.5 dS/m) salinity level (Figure 5.12). Otherwise, the addition of mycorrhizas tended to increase arbuscule formation under no salt condition (0 dS/m) and at the high (3.5 dS/m) salinity level (Figure 5.12).

Table 5.9: Summary of the results of Analysis of Variance of mycorrhizal physiological organ root association at different salinity levels (EC) (0, 1.5, and 3.5 dS/m) and AM treatment (Gm = *Glomus mosseae*, Ge = *G. etunicatum*, and the two combined). Degrees of freedom of salinity levels = 2, 58; Gm = 1, 58; Ge = 1, 58.

	Hyphae		Vesicles		Arbuscules	
	F-value	P-value	F-value	P-value	F-value	P-value
Salinity (EC)	0.96	0.39	1.4	0.3	0.91	0.41
Gm	3.1	0.08	0.15	0.7	1.5	0.23
Ge	1.5	0.22	0.01	0.93	0.00	0.95
Salinity (EC) x Gm	0.8	0.6	1.7	0.19	0.7	0.5
Salinity (EC) x Ge	2.0	0.14	3.7	< 0.05	0.3	0.8
Gm x Ge	0.2	0.7	5.5	< 0.05	0.8	0.4
Salinity (EC) x Gm x Ge	3.3	< 0.05	3.8	< 0.05	3.4	< 0.05

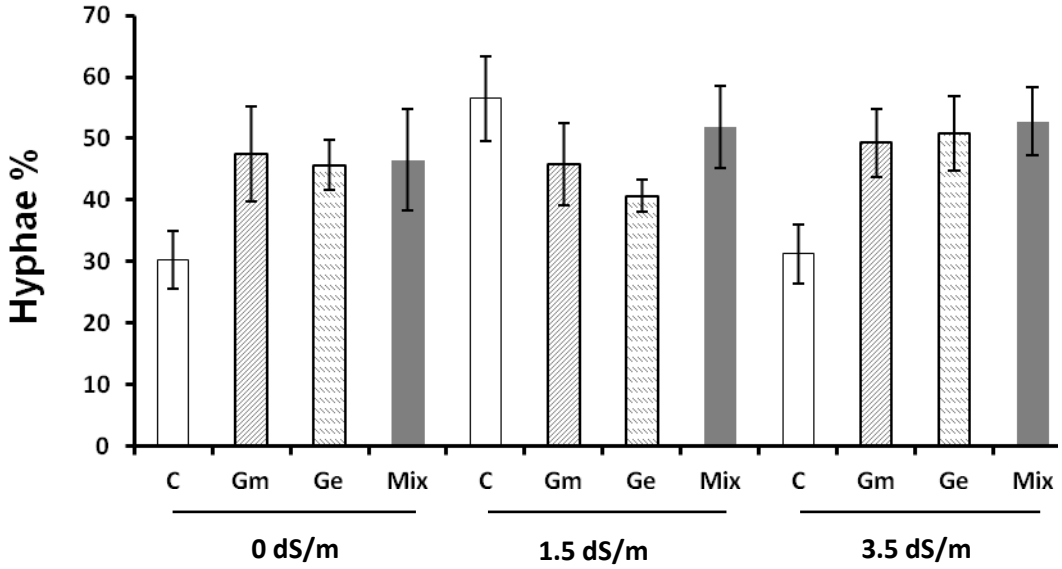

Figure 5.10: **Percentage of root colonisation by hyphae for different species of mycorrhizas under 0, 1.5, and 3.5 dS/m salt salinity levels. The mycorrhizal treatments were C, without mycorrhizal addition; Gm, *Glomus mosseae*; Ge, *G. etuicatum*; and Mix, a mix of the two species.**

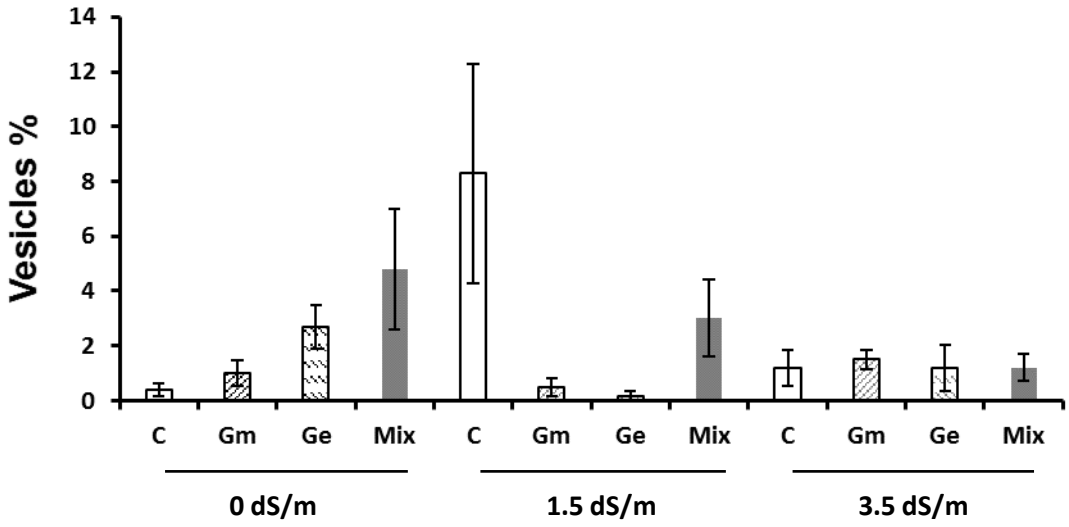

Figure 5.11: Percentage of vesicles colonisation of plant root at different salinity levels (0, 1.5 and 3.5 dS/m). The mycorrhizal treatments were C, without mycorrhizal addition; Gm, *Glomus mosseae*; Ge, *G. etunicatum* and Mix, a mix of the two species.

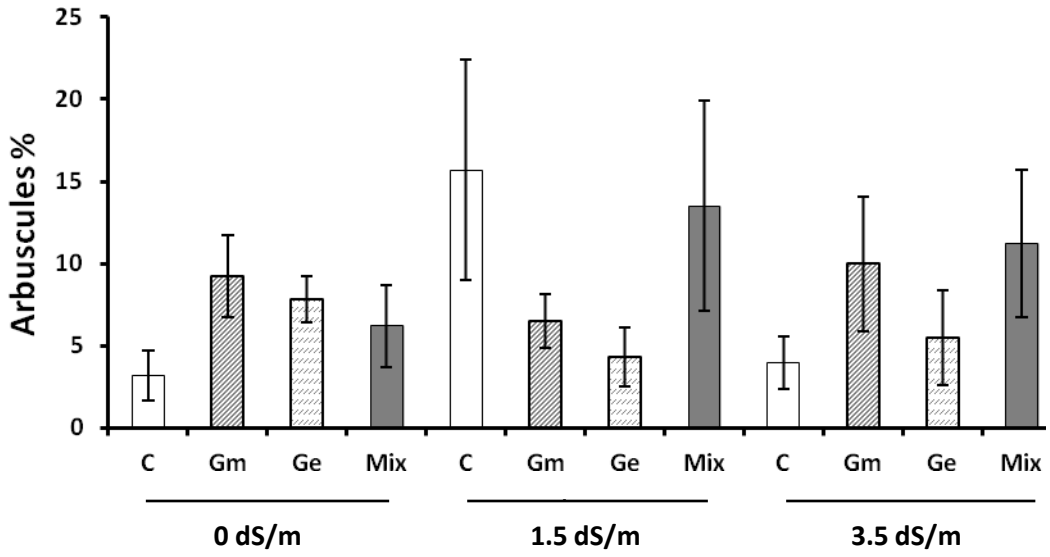

Figure 5.12: Percentage of arbuscules colonisation of plant root at different salinity levels (0, 1.5 and 3.5 dS/m). The mycorrhizal treatments were C, without mycorrhizal addition; Gm, *Glomus mosseae*; Ge, *G. etuicatum* and Mix, a mix of the two species.

5.4.3 Second generation

Salinity did not show any effect on second generation seed germination; however, there were different rates of seed germination with mycorrhizal inoculation and a significant interaction between the fungi (Table 5.10). The combined addition of both species of mycorrhizas was associated with a little increase in seed germination compared to individual AM fungi, which tended to reduce it slightly (Figure 5.13). On the other hand, the different mycorrhizal inoculation and salinity effect did not show any remarkable effects on seedling growth (Table 5.10).

Table 5.10: Summary of the results of Analysis of Variance for offspring seed germination and seedling length at different salinity levels (EC) (0, 1.5 and 3.5 dS/m) and AM treatment (Gm = *Glomus mosseae*, Ge = *G. etunicatum*, and a Mix of the two). Degrees of freedom of salinity levels = 2, 59; Gm = 1, 59; Ge = 1, 59.

	Seed germination		**Seedling length (cm)**	
	F-value	**P-value**	**F-value**	**P-value**
Salinity (EC)	2.7	0.08	0.9	0.4
Gm	0.09	0.76	0.00	0.97
Ge	0.03	0.86	0.3	0.59
Salinity (EC) x Gm	0.65	0.5	0.5	0.6
Salinity (EC) x Ge	1.2	0.32	0.4	0.7
Gm x Ge	4.1	< 0.05	0.04	0.85
Salinity (EC) x Gm x Ge	0.22	0.8	0.85	0.43

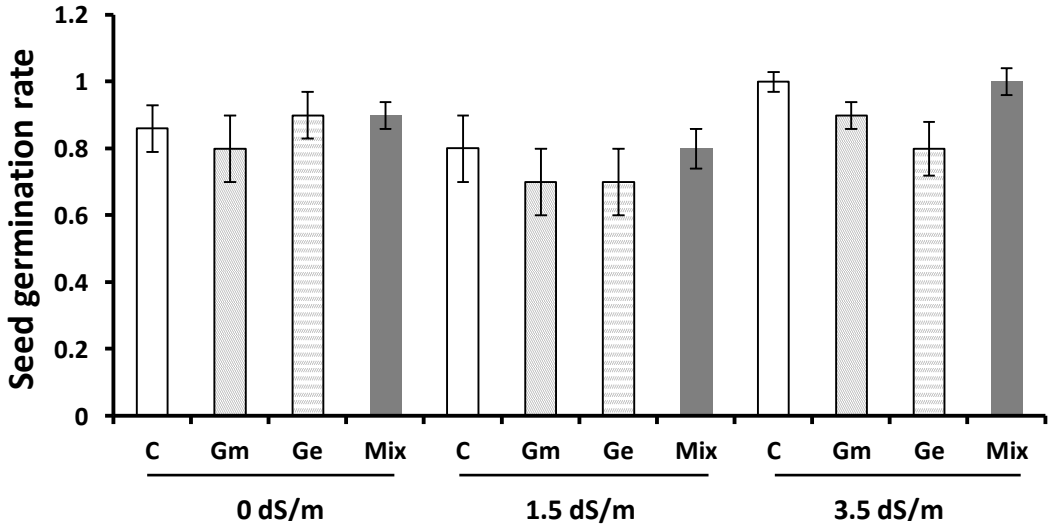

Figure 5.13: Seed germination rate for offspring whose parental plants were treated with salinity and different species of mycorrhizas. Ge, *G. etunicatum*; Gm, *G. mosseae*; Mix, adding G. *etunicatum* + *G. mosseae*; and C, control. Salt salinity levels (0, 1.5, and 3.5 dS/m).

5.5 Discussion

5.5.1 First field experiment with mixed commercial mycorrhizas

Mycorrhizal treatment under field conditions showed no remarkable effect on *Plantago* plant height under different salinity stresses. In other studies, mycorrhizal addition did not enhance the different growth parameters of the plant under salinity stress. Graham and Syvertsen (1989) in a study of the effect of *Glomus intraradices* on two species of citrus (sour orange and sweet orange) under salinity stress did not find any enhancement of different growth characteristics of the plants; instead, the roots of mycorrhizal plants under salinity accumulated more chlorine ions than the control. Others suggest that mycorrhizal colonisation can negatively affect plant growth under stress because of the carbon drain imposed on the roots (Snellgrove *et al.*, 1982; Koch & Johnson 1984). Root mycorrhizas under salinity stress demand more carbon from the root to overcome the stress (Hartmond *et al.*, 1987).

Colonisation of plants by mycorrhizas at different salinity levels did not have any effect on final plant dry biomass. It was indicated in previous studies that the addition of mycorrhizas may not enhance shoot biomass under salinity conditions. A

study of the response of cucumber (*Cucumis sativus* L.) under NaCl stress with different species of *Glomus* did not yield larger biomass under stress, and the control plants without the addition of any species of mycorrhizas had the larger mean (Rosendahl & Rosendahl 1991).

The length of inflorescences was also unaffected by the salinity treatments and mycorrhizal inoculation in our study. This finding contradicted a previous study by Bryla and Koide (1990) in which the inoculation of tomato (*Lycopersicon esculentum* Mill.) with mycorrhizas increased the fruit number. In contrast to the lack of effect of increased salinity levels on inflorescence head length, the number of inflorescences significantly decreased. Hence, the results for inflorescence number were in line with previous studies that found increasing salinity stress levels reduced plant production and crop yield (Mass 1986; Colla 2008).

The types of salts used in the experiment significantly affected some growth parameters, especially leaf number, shoot dry biomass, inflorescence number, and seed weight. The mixed salts type reduced the growth substantially in comparison with the NaCl used in this experiment. It was previously reported that by using different types of salts, such as NaCl, Na_2SO_4, $MgCl_2$, and $MgSO_4$ in clover (*Trifolium alexandrinum* L.) plants with mycorrhizas, NaCl significantly reduced the biomass and phosphorus uptake from the soil in comparison with the other saline ions (Gharineh *et al.*, 2009). Thus, different types of salts have different effects on salinity-plant interactions.

A previous study showed that with increasing salinity levels the mycorrhizal colonisation of plant roots decreased substantially (Kaya *et al.*, 2009). Salinity may prevent germination of AM spores (Hirrel 1981) and could make obstacles for hyphae to spread in the soil (McMillan *et al.*, 1998). Moreover, it was shown that stress reduces the number of arbuscules formed (Pfeiffer & Bloss 1988). In this experiment, however, the amount of mycorrhizal colonisation did not differ between different salt treatments. In some situations, different salinity stresses may not have effects on mycorrhizal colonisation (Mergulhao *et al.*, 2002). Another experiment by Chambers *et al.* (1980) also found that the addition of different levels of salinity did not have an effect on mycorrhizal colonisation behaviour in plant roots.

The results obtained in this experiment confirmed many previous observations that stressed mycorrhizal fungi produce more spores. At higher levels of salinity stress with both salt types, the mycorrhizas appeared to produce spores, a clear indication that the fungus was under some form of stress. Addition of salt to soil increases the pH,

which makes the soil more alkaline. A higher rate of mycorrhizal spore production was recorded for different species of mycorrhizas in neutral and alkaline environments than in acidic media (Green *et al.*, 1976). It was recorded that under stress mycorrhizas tend to produce more spores. In support of this notion, it has been shown that in the dry season when there is a lack of water mycorrhizas produce more spores than in the wet season with an abundance of water resources (Guadarrama & Alvarez-Sanchez 1999). Moreover, when plant roots decay or are stressed, the mycorrhizas also produce more spores (Redhead 1975). A large number of mycorrhizal spores were also found in areas contaminated with wastewater irrigation (Ortega-Larrocea *et al.*, 2001).

The germination of second-generation seedlings was successful and more frequent under the parental treatment with mycorrhizas than non-treated parents. Mycorrhizas enhance the uptake of nutrients in the mother plant tissues. Maternal plants with higher nutrients in their tissues may produce seeds with higher nutrient reserve concentrations, which lead to better seed germination and faster establishment of the second-generation plants (Aarssen & Burton 1990; Sills & Nienhuis 1995; Cheplick & Sung 1998). Furthermore, mycorrhizal inoculation of maternal plants improved their resistance to different environmental stresses, and they produced better quality seeds with better physiological changes, namely a thinner seed-coat formation, which allows greater water permeability and triggers the seed embryo to germinate faster under good conditions (Raven *et al.*, 1999).

5.5.2 Second field experiment with individual species of mycorrhizas

Regardless of small positive changes in plant height, different phenotypic parameters in the first generation did not show any significant changes with the addition of mycorrhizal treatments at all salinity levels. It seems that the different mycorrhizal species used in the experiments did not work under the field conditions to enhance plants' mineral acquisition and growth at different salinity stress levels. A similar experiment by Abbaspour (2008) on *Carthamus tinctorius* L. plants also showed that the addition of *G. etunicatum* mycorrhizas at different salinity levels did not enhance the absorption of nitrogen, an essential element for plant growth, and better establishment of different plant parts. Likewise, a study on the effect of salinity on orange plants using *G. intraradices* revealed no enhancement of mycorrhizas for sour

orange (*Citrus aurantium* L.); with sweet orange (*C. sinensis* Osb.), the mycorrhizas resulted in accumulation of chlorine anions at the expense of phosphate (Graham & Syvertsen 1998). Comparable findings by Buwalda *et al.* (1983) demonstrated that mycorrhizal spring wheat and winter barley accumulated more chlorine anions in their tissues than the essential microelements for growth.

At the plant species level, it has been found that different sub-species may have different responses to salinity stress (Shannon & Grieve 1999). It is possible that the type of *Plantago* used in the experiment did not react positively with the mycorrhizal species used under salinity stress. Similar results were obtained on the effects of different salinity stress levels on rootstocks of grape (*Vitis* spp) (Belew *et al.*, 2010). The growth parameters of four different cultivars of grape responded differently under salt stress with inoculation of *G. fasciculatum* mycorrhizal species.

In the current field experiment, at the control level (without the addition of salt to stress the plant) the mycorrhizal addition to the plant did not affect the growth parameters in the first generation and did not change the second-generation growth parameters positively or negatively. The same result was found by Al-Karaki (2006) for the effect of *G. mosseae* interaction with soil salinity on tomato yield. The *G. mosseae* mycorrhiza did not affect the fruit weight of tomato under the non-saline conditions, but with the addition of salinity the mycorrhizas enhanced the fruit weight (Al-Karaki 2006).

Mycorrhizal species of both *G. mosseae* and *G. etunicatum* failed to react positively with the plant association to overcome salinity stress. It is known that different mycorrhizal species have different abilities to influence plant growth under saline conditions. A survey of different mycorrhizal species designed to help *Prosopis juliflora* Swartz. grow under saline conditions in coastal areas of India indicated that out of 16 different species of mycorrhizas, only *G. macrocarpum, G. fasciculatum*, and *Scutellospora corralloides* helped the trees resist soil salinity and yield better biomass (Selvaraj & Kim 2004). Also, it seems that the mycorrhizal species used in this experiment did not react under the salinity condition, probably because they originated from a non-saline environment. Salinity experiments on different citrus tree species confirmed that mycorrhizas (*G. mosseae*) native to desert soil with a high level of salt performed better under salinity irrigation solution than AM originating from areas of low soil salinity (Levy *et al.* 1983). In the current study, it was decided not to try to use mycorrhizas originating from saline conditions. This is because the overall aim of this

thesis is to test whether commercially available strains can mitigate salt stress in plants. If one has to first isolate fungi from saline soils, culture, and bulk up the inoculums, this is something that would require a vast financial undertaking, which was beyond the scope of this study.

The addition of salt did not negatively affect the rate of mycorrhizal colonisation of plant roots, as was expected. In some plant species, the addition of salts does not reduce the rate of mycorrhizal colonisation of plants, and in other situations with increasing salinity levels the AM fungi-root association increased. For example, experiments on the rate of colonisation of native soil AM fungi on two annual sunflowers (*Helianthus paradoxus*) showed that colonisation increased with addition of higher salinity levels (Van Auken & Freidrich 2006). Moreover, a study on the mycorrhizal colonisation of leguminous *Strophostyles helvola* L. Ell under higher concentrations of soil salinity revealed that the hyphal structure of mycorrhizas increased rather than decreased (Tsang & Maun 1999).

Results of the current experiment did not show a substantial improvement of mycorrhizal association with plants to overcome salinity stress, perhaps because the experiment was performed under field conditions with multiple impacts from various environmental factors. For example, some experiments showed that mycorrhizas decreased the rate of root colonisation when the soil was supplied with phosphorus (Mendoza & Pagani 1997; Cornwell *et al.*, 2001). On the other hand, a study on mycorrhizal association in grassland indicated that phosphorus or other nutrients did not have any effect on the length of root colonisation (Sanders & Fitters 1992). Under field conditions, the weather patterns in different seasons can affect the performance of the mycorrhizal-plant association in different ways. *Lotus tenuis* Waldst. & Kit. mycorrhizal colonisation reached the highest level during spring and summer; in contrast, *Paspalum vaginatum* Sw. had higher colonisation interaction with mycorrhizas during autumn and winter (Garcia & Mendoza 2008). Also, dry and moist soil cycles affect the spores of mycorrhizas in terms of germination and association with the plant roots during different seasons in field situations (Rickerl *et al.*, 1994). Moreover, many previous experiments in open grassland indicated that the types of soil and different plant species have different results with association with soil AM (Allen *et al.*, 1995; Muthukumar & Udaiyan 2002; Escudero & Mendoza 2005).

5.6 Conclusion

The results obtained from both field experiments showed that mycorrhizas did not actually help the plant overcome salinity at higher stress. Also, different levels of salinity and different salt types influenced mycorrhizal species interaction with plants in different ways. In the first field experiment with higher salinity levels and mixed commercial mycorrhizas, the results were impressive regarding the plant offspring quality. In the second experiment with reduced levels of salinity and the addition of individual mycorrhizal species, the results were not conclusive. Thus, it is recommended that the second field experiment be repeated under controlled conditions for comparison of results.

Chapter 6

The interaction of arbuscular mycorrhizal fungi and *Plantago lanceolata* under salinity stress in glasshouse and controlled room environments

6.1 Introduction

Arbuscular mycorrhizal **(**AM) fungi occur in all soil types, and can enhance plant responses to different types of environmental stress (Allen & Boosalis 1983). A considerable body of evidence indicates that AM fungi can help plants survive and overcome saline stress (Diallo *et al*., 1999; Burke *et al*., 2003; Tain *et al*., 2004). Even when seawater is used for the irrigation of mungbean plants (*Vigna radiate* L. Wilczik), those colonised by AM fungi show augmented growth compared to uncolonised plants (Rabie 2005). AM colonisation has been shown to enhance the ability of plants to combat salt ions by improving the absorption of nutrients (Zandavalli *et al*., 2004), altering plant physiology and osmotic regulation for improved adaptation to salinity stress (Roa & Tak 2002), as well as enhancing photosynthesis (Mergoguihae *et al*., 2002). Mycorrhizas also increase the activities of antioxidant enzymes to protect the plant's internal tissues from damage caused by stress (Talaat & Shawky 2011).

Other abiotic factors that can play a role in soil salinity-plant interactions include soil type, water quality and climatic conditions. Maas (1993) stated that climate was the most important factor affecting plant-salinity stress relationships. Plants under salinity stress can sustain growth if the weather is humid and cool, but if the climate is hot and dry, salinity will be very stressful for the plant. Also, plant responses to AM colonisation can vary according to the surrounding environment, whether in controlled situations or in the field. Experiments in a glasshouse showed that various species of AM fungi affect plant growth differently, depending on the temperatures at which the experiments are performed (Smith & Roncadori 1986). However, a study of different AM species colonising plants under laboratory and field conditions suggested that the AM species acted in the same way regardless of the surrounding environment (Pringle & Bever 2008). Thus, the impacts of the surrounding environment on AM-plant interactions can yield different effects on plant growth, and should not be excluded when studying AM-plant interactions.

The aim of the first experiment was to investigate the association between commercial mycorrhizal fungi and plants under controlled room conditions (CER) at lower salinity stress levels. It was hypothesised that AM fungi would successfully associate with the plant at lower salinity stress. The aim of the second experiment was to investigate the effectiveness of mycorrhizal association with plants during saline stress under glasshouse conditions. It was hypothesised that in the glasshouse, where the temperatures and evapotranspiration were increased, the AM fungi would be less efficient in helping *P. lanceolata* overcoming soil salinity.

6.2 Materials and methods

Plantago lanceolata (section 2.6.1) seeds were germinated under CER conditions (section 2.1). After the seedlings had reached the four-leaf stage, they were transferred into individual 11 cm square pots filled with commercial sterilised compost (section 2.1.3). Half of the plants were inoculated with commercial AM fungi (section 2.2.1) at the root level before planting in the pots to produce a treatment colonised with AM fungi.

6.2.1 Mixed salts and a commercial mix of arbuscular mycorrhizal fungi in the controlled environment room (experiment 1)

A total of 48 *P. lanceolata* plants was selected for this experiment of which 24 plants were inoculated with commercial AM fungi and the other 24 were untreated (section 2.2.1). For salinity stresses, four levels of salinity treatment were used, these were: 1 dS/m, 1.7 dS/m, 2.2 dS/m electrical conductivity and tap water as the control (Appendix B). Each level was assessed with 12 plants, 6 with the AM treatment and 6 without. Each week, 100 ml of the respective solution was applied to each plant in each treatment group to keep the required salinity level in the pot constant. Nutrient solution (section 2.1.4) was added to the plants at two-week intervals. The experiment was conducted in the controlled environment room (section 2.1).

The duration of the experiment was four months and at the end of this period plant height, leaf number, inflorescence number and length were measured. Inflorescence weight was also measured as an indication of seed weight. Initial shoot

biomass was recorded for each plant and final dry shoot weight was measured for each treatment (section 2.4). Roots for each plant were cleaned and stained for AM visualisation (section 2.5.1). The stained roots were prepared on glass slides for AM fungal quantification and identification of the different structures such as vesicles, hyphae and arbuscules (section 2.5.2).

The seeds produced by the F1 generation plants grown under the different salinity treatments were used to determine the germination rate of F2 generation plants. From each F1 plant, 15 healthy seeds were selected for the germination test. Petri dishes (90 mm) containing filter paper were used for the germination test under a constant room temperature of approximately (26°C). The seeds of each F1 plant were divided across three Petri dishes, so that each dish contained five seeds. The seeds were watered daily with distilled water and daily seedling germination recorded for 7 days. At this point total shoot and root length were recorded for each successfully germinated seedling.

All data were tested for normality prior to performing a two-way ANOVA, employing salt and AM fungal inoculation as main effects using the statistical package Unistat version 6.0. Mycorrhizal colonisation was analysed by average percent of the means.

6.2.2 Mixed salts and a commercial mix of arbuscular mycorrhizal fungi under glasshouse conditions (experiment 2)

A total of 36 plants were selected, 18 of which were inoculated with the commercial AM fungi (section 2.2.1) and the remaining 18 plants (without AM inoculation) used as controls. Three salt treatments were used, 1.5 dS/m, 3.5 dS/m electrical conductivity and tap water (0 dS/m) as the control (Appendix B), with 12 replicate plants of each treatment (6 inoculated with the AM fungal mix and 6 untreated). Each week, 100 ml of the respective salt solution was applied to each plant to maintain the required salinity level in the pot constant. Nutrient solutions (section 2.1.4) were added to the plants at two-week intervals.

The experiment was performed in a glasshouse, at a day-time temperature of 25-30°C, a minimum night-time temperature of 10°C and the humidity maintained at 50 % during the experiment. The photosynthetic photon flux density (PPFD) was 500-750 µmol photon m^{-2} sec^{-1} with a 16/8 h light/dark cycle. The rest of the experimental set up

and analyses were identical to those reported in the controlled environment room experiment (section 6.2.1).

6.3 Results

6.3.1 Mixed salts and a commercial mix of arbuscular mycorrhizal fungi in the controlled environment room (experiment 1)

Commercial AM fungal inoculation had no significant effects on plant growth parameters in the first generation for controls or plants subjected to saline stress (Table 6.1).

Table 6.1: Summary of the results of Analysis of Variance of plant growth parameters from first generation *Plantago lanceolata* grown under different salinity levels (1, 1.7 and 2.2 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi. Degrees of freedom for salinity levels = 3, 40 and for AM = 1, 40.

Parameters	EC		AM		EC x AM	
	F-value	P-value	F-value	P-value	F-value	P-value
Plant height (cm)	2.6	0.07	3.2	0.08	0.76	0.52
Leaf numbers	2.2	0.12	0.18	0.67	0.4	0.75
Shoot dry biomass (g)	1.2	0.34	0.75	0.4	0.3	0.9

In terms of reproductive plant structures, AM inoculation had no effect on inflorescence number, though this parameter was significantly affected by salt (Table 6.2). The plants grown at the 1 dS/m salt concentration produced the highest number of inflorescences compared to control and other salinity levels (Figure 6.1). The average inflorescence head length was not affected by salinity or the addition of AM fungi, and was similar for all treatments (Table 6.2). Seed weight increased significantly with the addition of salt but only up to a level of 1 dS/m (Table 6.2; Figure 6.2). However, there was also a significant interaction term seen between salt and AM inoculation (Table 6.2). This was because the AM effect was strong at high levels of salt, but was not seen at intermediate salt levels (Figure 6.2).

Table 6.2: Summary of results of Analysis of Variance of reproductive parts from first generation *Plantago lanceolata* grown under different salinity levels (1, 1.7 and 2.2 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi. Degrees of freedom for salinity levels = 3, 40 and for AM = 1, 40.

Parameters	EC		AM		EC x AM	
	F-value	P-value	F-value	P-value	F-value	P-value
Inflorescence number	5.5	< 0.001	0.21	0.65	1.2	0.29
Average inflorescence head length (cm)	0.75	0.53	0.08	0.77	1.9	0.15
Seed weight (g)	5.2	< 0.001	0.11	0.74	3.2	< 0.05

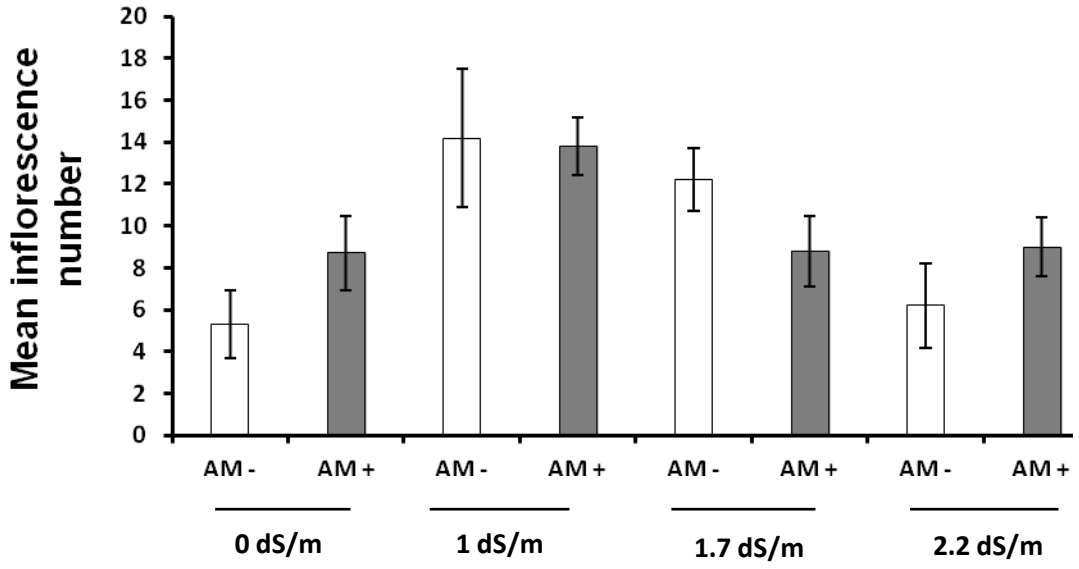

Figure 6.1: Number of inflorescences (mean ± SE) produced per plant by *Plantago lanceolata* when grown under different salinity treatments (dS/m) and inoculated with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).

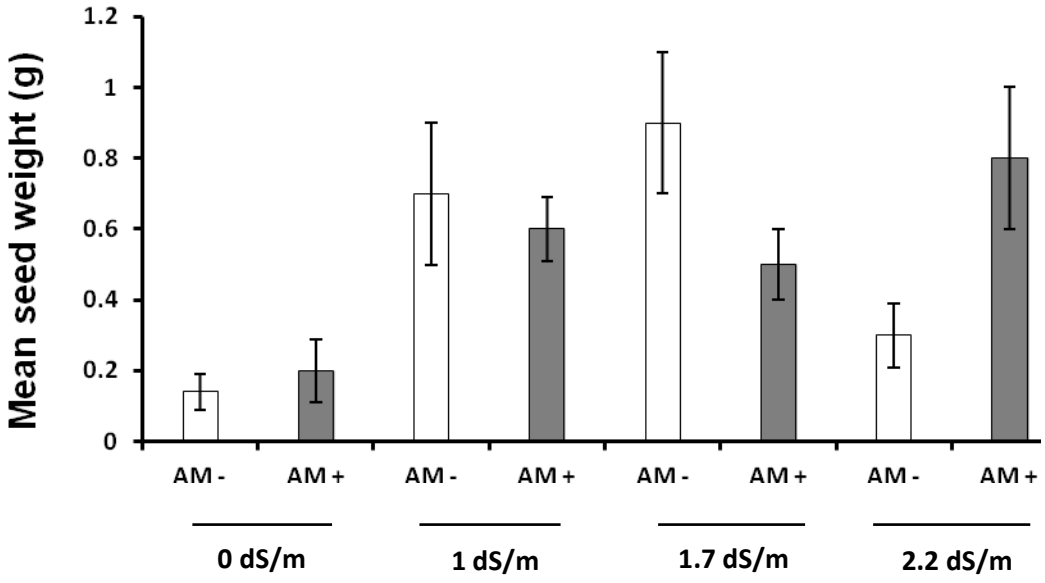

Figure 6.2: Total seed weight (g) (mean ± SE) per *Plantago lanceolata* plant when grown under different salinity treatments (dS/m) and inoculated with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).

Germination studies on second-generation seedlings revealed significant effects of salt addition on both germination and seedling length (Table 6.3), while there was no effect for AM addition on seedling germination and establishment. The salinity levels of 1 and 1.7 dS/m produced significantly higher rates of germination and seedling length compared to control and 2.2 dS/m salinity on parent plants (Figures 6.3 and 6.4). After root staining, no mycorrhizas were detected in any plants, even in those that had been inoculated.

Table 6.3: Summary of results of Analysis of Variance of second-generation *Plantago lanceolata* seedling germination and length when parent plants were grown under different salinity levels (1, 1.7 and 2.2 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi. Degrees of freedom for salinity levels = 3, 40 and AM = 1, 40.

Parameters	EC		AM		EC x AM	
	F-value	P-value	F-value	P-value	F-value	P-value
Seed germination rate	3.2	< 0.05	2.6	0.11	0.98	0.41
Length of the seedling (cm)	7.1	< 0.01	1.6	0.21	2.5	0.07

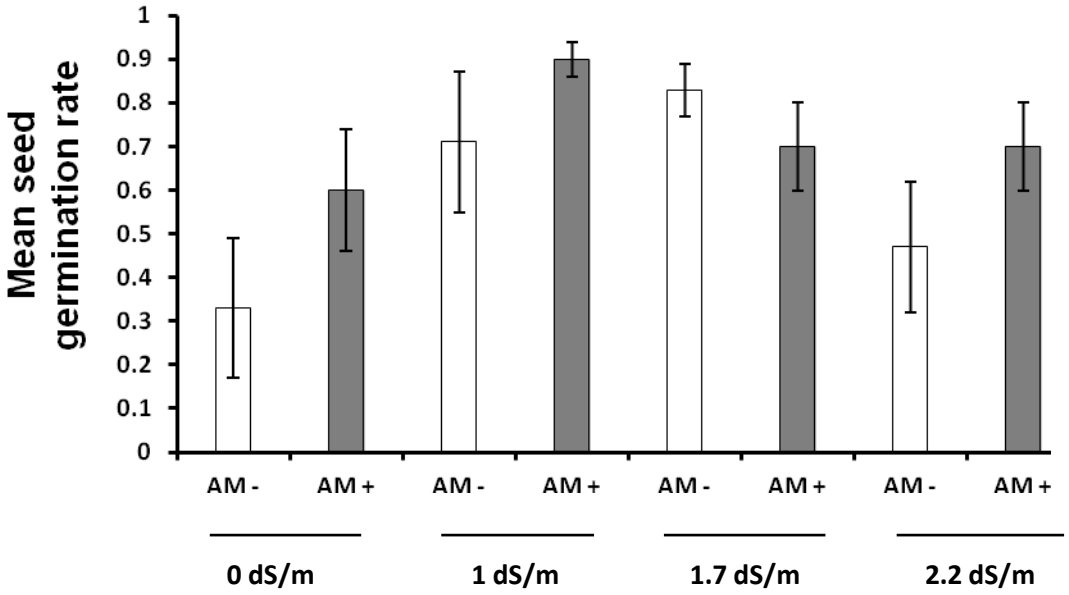

Figure 6.3: Second-generation *Plantago lanceolata* mean seedling germination rate (%) after the parent plants were grown under different salinity levels (0, 1, 1.7 and 2.2 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).

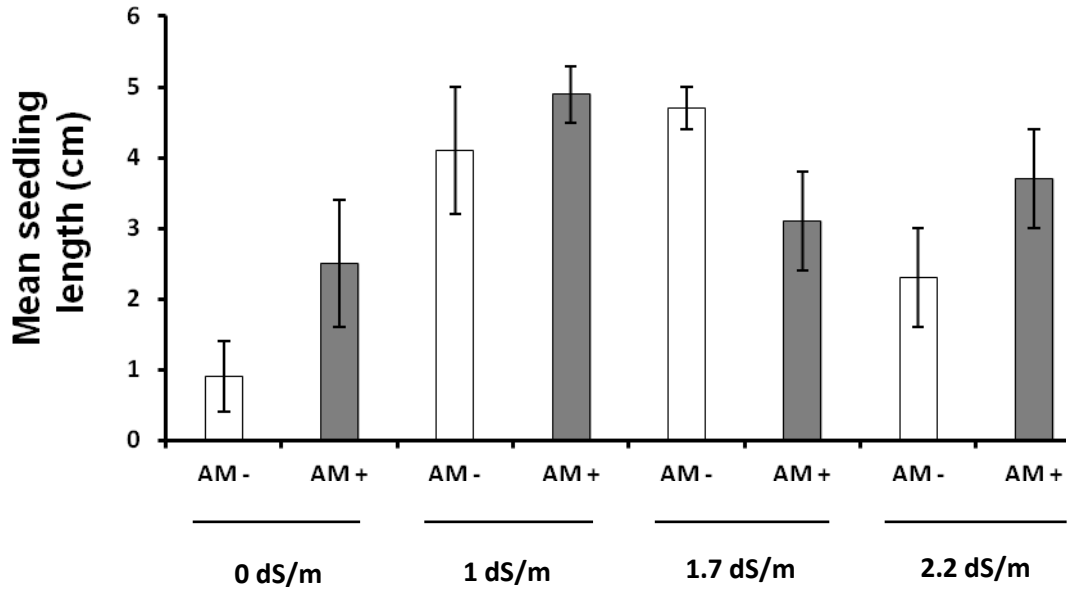

Figure 6.4: Second-generation *Plantago lanceolata* mean seedling length (cm) after 7 days growth under lab condition. The parental plants were grown under different salinity levels (0, 1, 1.7 and 2.2 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).

6.3.2 Mixed salts and a commercial mix of arbuscular mycorrhizal fungi under the glasshouse condition (experiment 2).

Plant growth parameters from the first generation *Plantago lanceolata* plants grown under different treatments varied (Table 6.4). Plant height was not affected by salt or AM addition, but there was a significant interaction between treatments. AM addition reduced height at intermediate salt levels, but increased height at high salt concentration (Figure 6.5).

Table 6.4: Summary of the results of Analysis of Variance of different plant growth parameters from first generation *Plantago lanceolata* grown under three salinity levels (0, 1.5 and 3.5 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi. The degrees of freedom for salinity levels = 2, 29 and for AM = 1, 29.

Parameters	EC		AM		EC x AM	
	F-value	P-value	F-value	P-value	F-value	P-value
Plant height (cm)	2.5	0.1	0.3	0.6	5.4	< 0.05
Leaf number	3.5	< 0.05	5.1	< 0.05	3.1	< 0.05
Shoot dry biomass (g)	4.2	< 0.05	0.3	0.6	0.9	0.4

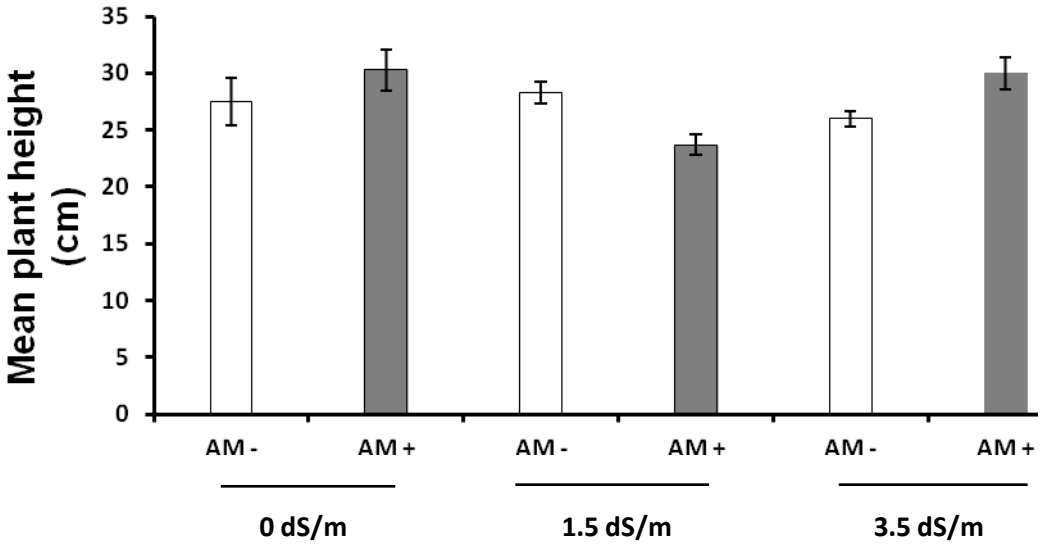

Figure 6.5: Mean height (cm) of *Plantago lanceolata* grown under different levels of salinity stress (0, 1.5 and 3.5 dS/m) and with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).

With leaf number, both salt addition and AM fungi significantly affected this parameter. High salt levels tended to reduce leaf number, while AM colonisation increased it. This was most apparent at the intermediate salt level, leading to a significant interaction term between the treatments (Figure 6.6).

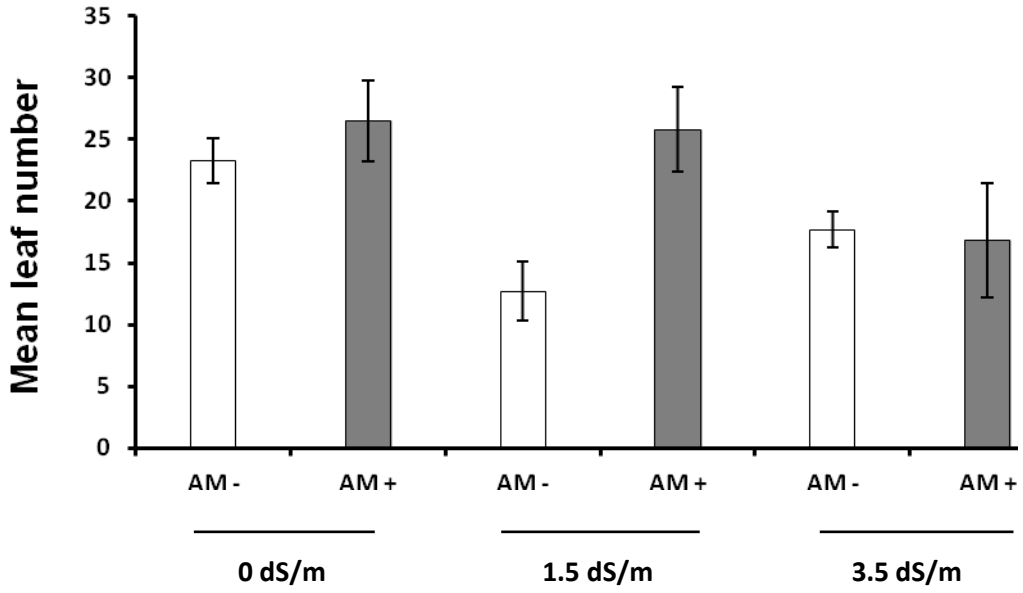

Figure 6.6: Mean leaf number of *Plantago lanceolata* grown under different salinity levels (0, 1.5 and 3.5 dS/m) and with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).

With shoot dry biomass, the addition of AM did not enhance plant growth either with or without salt addition (Table 6.4). Meanwhile, shoot dry biomass was significantly reduced at all salt levels compared with control plants (Figure 6.7).

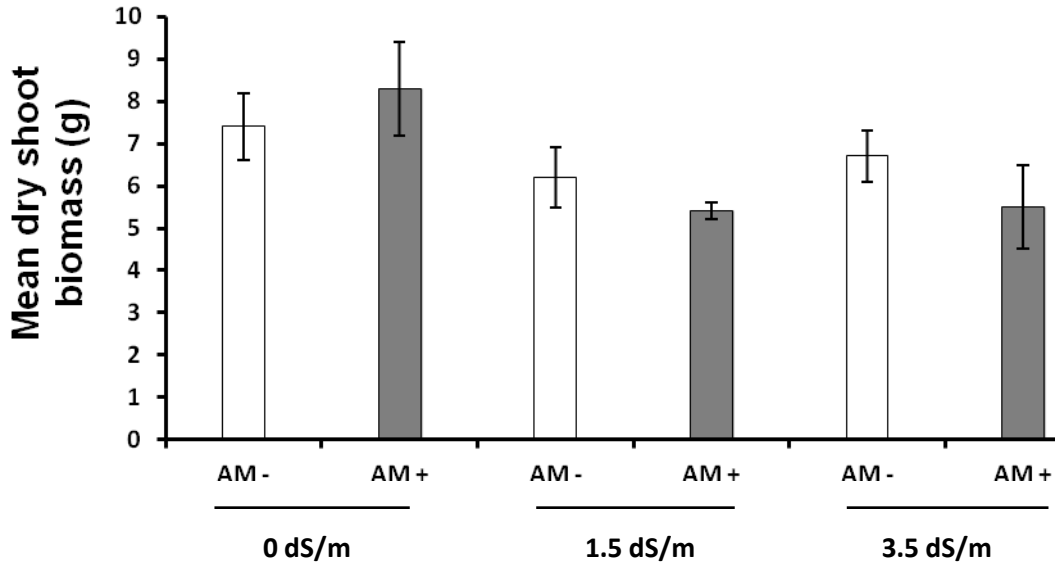

Figure 6.7: Mean dry shoot biomass (g) of *Plantago lanceolata* grown under different salinity levels (0, 1.5 and 3.5 dS/m) and with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).

Salt or AM addition had no effect on inflorescence number or length (Table 6.5). However, salinity and AM colonisation significantly reduced the total weight of seeds produced (Table 6.5; Figure 6.8).

Table 6.5: Summary of results of Analysis of Variance of reproductive parts from first generation *Plantago lanceolata* grown under different salinity levels (0, 1.5 and 3.5 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi. The degrees of freedom for salinity levels = 2, 29 and for AM = 1, 29.

Parameters	EC		AM		EC x AM	
	F-value	P-value	F-value	P-value	F-value	P-value
Inflorescence number	2.9	0.08	0.2	0.6	2.5	0.1
Average inflorescence head length (cm)	0.05	0.95	1.6	0.2	0.8	0.5
Seed weight (g)	4.0	< 0.05	8.5	< 0.001	1.5	0.2

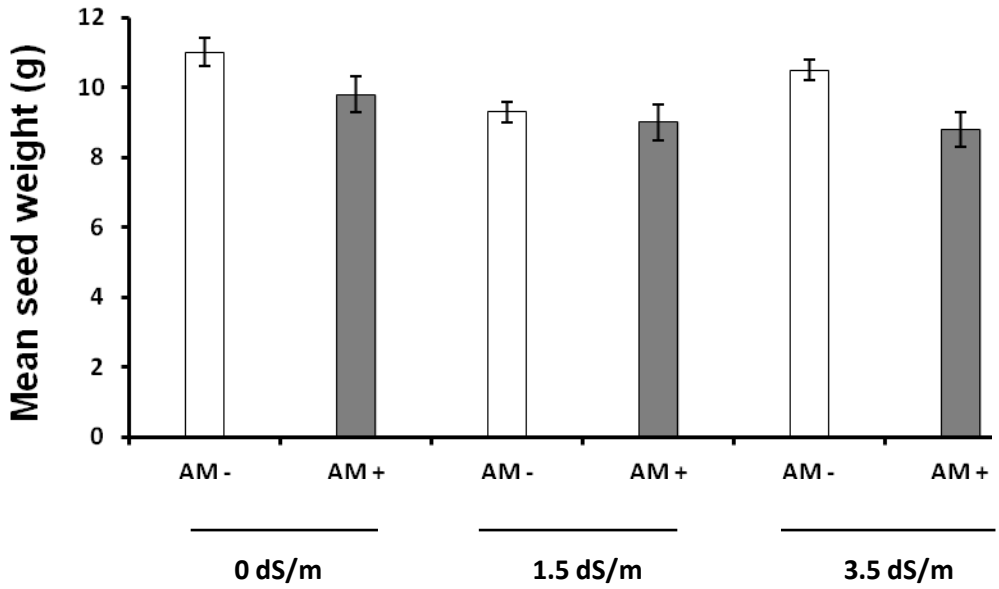

Figure 6.8: **Total seed weight (g) (mean ± SE) per *Plantago lanceolata* plant when grown under different salinity treatments (0, 1.5 and 3.5 dS/m) and inoculated with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).**

Root staining for AM colonisation was successful and there was no indication that addition of salt affected the AM structures (Table 6.6). In general, plants showed colonisation by hyphae, but levels of vesicle and arbuscular colonisation were very low. Plants not inoculated with AM fungi did not display colonisation.

Table 6.6: Mean percentage root length colonisation of the different arbuscular mycorrhizal structures in *Plantago lanceolata* grown under different salinity levels (0, 1.5 and 3.5 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi (+AM) or not (-AM).

Treatments	Hyphae %	Vesicles %	Arbuscules %
0 EC + AM	18	0	1
0 EC – AM	0	0	0
1.5 EC + AM	9	0	1
1.5 EC – AM	0	0	0
3.5 EC + AM	11	0	1
3.5 EC - AM	0	0	0

No significant differences in the rate of seed germination or seedling length after 7 days of germination (Table 6.7) were seen for second-generation plants. Neither the addition of salt nor AM colonisation affected the germination rate or seedling one week after germination.

Table 6.7: Summary of results of Analysis of Variance of seed germination and seedling growth in second-generation *Plantago lanceolata* plants, where the parents plants were exposed to different salinity levels (0, 1.5 and 3.5 dS/m EC) and inoculated with a commercial mix of arbuscular mycorrhizal fungi. The degrees of freedom for salinity levels = 2, 29 and for AM = 1, 29.

Parameters	EC		AM		EC x AM	
	F-value	P-value	F-value	P-value	F-value	P-value
Seeds germination rate	0.52	0.6	0.9	0.3	1.8	0.17
Seedling length after germination	0.8	0.45	0.05	0.8	0.05	0.9

6.4 Discussion

6.4.1 Mixed salts and a commercial mix of arbuscular mycorrhizal fungi under the controlled environment room experiment

First-generation plant vegetative parameters (height, leaf number and dry shoot biomass) were not significantly influenced by the mild salinity levels under CER conditions. The salinity stress levels in this particular experiment were 2.2 dS/m and below, so the lack of severe detrimental effects on plant growth was perhaps not surprising. AM-inoculated plants did not differ in any respect from non-inoculated counterparts, a finding that could be explained by the lack of AM colonisation after root staining at the end of the experiment. It is possible CER conditions were unfavourable to the establishment of an association between AM fungi and plant roots even at low salinity levels. Examining the negligible response to light salinity stress, a previous study on leguminous plants showed that low saline stress did not severely affect vegetative growth, but at medium salinity (5 dS/m) and above, there was a substantial decrease in growth (Khan *et al.*, 1999). Additionally, light salinity levels had no

negative effect on the growth of mulberry (*Morus alpa* L.), whereas at 8 dS/m, significant growth suppression was observed (Agastian *et al.*, 2000). Testing the performance of different rice varieties under saline stress also showed that above 3.5 dS/m, the growth organs started to decline severely, leading to death (Zeng & Shannon 2000). Hasegawa *et al*. (2000) reported that plants tend to respond to salinity stress above 2 dS/m electrical conductivity, below which only minor changes occur (depending on the plant type).

Reproductive output was affected by light salinity stress, where certain reproductive parameters in *P. lanceolata* (inflorescence number, seed weight, F2 seed germination rate and seedling length) were increased at 1 dS/m when compared to controls (0 dS/m). Certain plant species have similarly shown improvements at low salinity levels, such as the seed germination rate of cotton (*Gossypium hirsutum* L.), which increases at low salinity treatment compared to control (Jalaludin 1993). The effects of salinity were examined with various soybean cultivars (*Glycine max* L. Merrill), which indicate height, root length, root dry weight and leaf dry weight under salinity stress increase for the Mancon cultivar, while the Irigious soybean cultivar shows increases in shoot height when treated with low levels of salt (Tuncturk *et al*., 2008).

Physiologically speaking, salt ions (especially Na^+) tend to accumulate in vacuoles under certain salinity stress as a mechanism to prevent plant deterioration, which increases plant size (Mimura *et al.*, 2003). As such, the salt tolerant plant *Arabidopsis thaliana* shows an accumulation of salt ions in the vacuole (Gaxiola *et al*., 1999). The accumulation of ions in vacuoles helps plants overcome salinity by shifting the ionic toxicity for the cytoplasm and increasing cellular osmolality to withstand osmotic stress. It would be tempting to speculate the increased size of *P. lanceolata* under salt stress was not related to growth factors, but rather a part of a salt tolerance mechanism.

Second-generation *P. lanceolata* plants demonstrated enhanced seedling germination at the light salinity level (1 dS/m) compared to the non-saline control. The improved quality of seed production under certain salinity stress has been attributed to ethylene production (Silva *et al.*, 2014). A very recent study by Silva *et al.* (2014) on three different species of tropical forage legumes demonstrates that Brazilian stylo (*Stylosanthes guianensis* Aubl.) has an improved seed output than other species due to an increased rate of ethylene biosynthesis. Moreover, it was proposed that under salinity

stress, ethylene and glutamate bind to increase seed germination (Chang *et al.,* 2010). Ethylene has also been suggested to interact with several hormones (such as polyamines and brassinosteroids) under salinity stress to enhance seed quantity and germination (Zapata *et al.,* 2003; Wang *et al.,* 2011). In the family Brassicaceae, ethylene has been shown to speed endosperm rupture during seed germination under environmental stress (Linkies *et al.,* 2009; Linkies & Leubner-Metzger 2012). Even though ethylene gas was previously considered a growth suppressor (Abeles *et al.,* 1992), under salinity stress, it can serve as a growth promoter (Pierik *et al.,* 2006).

Even when salinity levels were reduced below 2 dS/m, mycorrhizas still failed to colonise the plant roots, which suggested other factors affecting plant-mycorrhizal interactions existed (other than salinity). It is possible that spores of the commercial mycorrhizal fungi went into a long dormancy period because of product storage. Tommerup (1983), in studies on breaking spore dormancy for different species of mycorrhizal fungi, found that certain mycorrhizal fungi become dormant for 6 months without successful association with plant roots. A study by Judge *et al*. (2002) on *Glomus intraradices* showed that storage conditions can play a major role in spore dormancy and prevent successful colonisation. In addition to the impact of storage conditions on mycorrhizal quality, there are other edaphic factors that are important for successful mycorrhiza–plant associations. For example, low soil pH can interfere with spore germination and prevent the association of hyphae with roots (Clark 1997) and trace amounts of certain heavy metals can prevent mycorrhizal interaction with plants (Marschner 1991). In certain plant species, chemical inhibitors of non–host mycorrhizal species can be released as well (Vierheiling & Ocambo 1990), and among these inhibitors, alkenyl glucosinolate is the main chemical which inhibits mycorrhizal spore germination in some plants roots (Larsen 1981). Apart from environmental or soil factors, it is possible the *Plantago* plants used in the experiment were incompatible with the mycorrhizal species used in this study, and the fungi failed to associate.

6.4.2 Mixed salts and a commercial mix of arbuscular mycorrhizal fungi under glasshouse condition experiment

The plant-AM symbiosis was studied in a glasshouse environment using a different range of salinity levels to gain insights into the behaviour of the fungi under various

experimental settings. Plant leaf number and dry biomass were significantly decreased with increasing salinity levels, and saline stress is known to decrease plant vegetative growth and undermine the propagation of certain plant parameters. Increasing levels of proline are found in plant tissues with increasing salinity levels (Pujol *et al*., 2001; Parida *et al*., 2002), and proline accumulation under conditions of salinity indicates a stress reaction in plant species (Wang *et al*., 2004).

Mycorrhizal associations in this experiment were highest in the control (with no salt addition) and decreased with increasing salinity levels. Increasing soil salinity reduces mycorrhizal colonisation of the root due to inhibition of spore germination (Hirrel 1981), and the growth of hyphae is reduced in saline conditions, diminishing colonisation (McMillen *et al*., 1998). The ability of hyphae to absorb phosphate from the soil and deliver it to the root is substantially reduced under saline conditions, which can affect root metabolic rate and reduce mycorrhizal development (Juniper & Abbott 2006; Sheng *et al*., 2008).

Seed weight was reduced in plants inoculated with AM fungi. AM colonisation may not always increase seed weight, as shown by Nuortila *et al*. (2004) where AM inoculation of *Campanula rotundifolia* L. did not enhance seed weight. Similarly, Jensen (1993) found that AM fungal colonisation had no effect on seed weight or the number produced by *Hordeum distichon* L.

Plant reproductive parameters (namely inflorescence number and average inflorescence size), as well as second-generation seedling growth and germination did not change with the various parental treatments. Two factors may explain the ability of first-generation plants and offspring to resist changes in salinity stress. The first is that salinity levels used in this experiment (1.5 & 3.5 dS/m) were low and did not exert the detrimental effects seen at higher levels, and the second, that the experiment was done under glasshouse conditions where there was a considerably more light intensity than those in the CER study. Higher light levels lead to greater stomatal conductance and a higher net photosynthetic rate (Sheng 2008), resulting in increased photosynthesis and gas exchange, which may increase plant resistance to both salinity stress levels.

Successful colonisation by commercial AM fungi was seen in this study. It is tempting to speculate that under glasshouse conditions, more light would have been provided directly from the sun, increasing photosynthesis and allowing plants to allocate more carbon to the root, thereby stimulating the growth of mycorrhizal hyphae. In a previous study, *Gigaspora coralloidea* spores germinated better under full light

conditions than at lower light intensity (Schenck *et al*., 1975). Graham *et al*. (1982) showed that reducing the light caused less secretion of sugar from the root cells, which reduced the AM association with the Troyer citrange plant (*Poncirus trifoliate* L. Raf x *Citrus sinensis* L. Osbeck) and decreased the effect of beneficial symbiosis. Moreover, several studies have confirmed that under low light intensities, AM fungi do not associate strongly with plants, reducing their colonisation of the host (Hayman 1974; Daft & El Giahmi 1978; Son & Smith 1988). It appears that light intensity is a crucial factor determining successful colonisation of *Plantago* with the commercial mycorrhizal fungi used. As a result of the findings reported here, the effect of light intensity will have to be considered in future experiments.

6.5 Conclusion

The findings presented here showed that salinity levels and the microclimate play an important role in directing AM fungal interactions with plants, as the amount of light provided may have played a role. In future studies on the behaviour of mycorrhizal fungi under saline stress, the experimental setup and the amount of light provided to the plants should be considered. After using mixed commercial species of mycorrhizas in these experiments, it would be interesting to investigate the effects of using individual species of different mycorrhizal fungi (Chapter 7) to gain insights into their behaviour under salinity stress.

Chapter 7

An assessment of the effect of arbuscular mycorrhiza fungi in plants under salinity stress

7.1 Introduction

It has been estimated that about 6% of land across the globe is affected by saline soil accumulation, which represents about 800 million hectares of irrigable land (FAO 2008). The problem of salinity is rapidly spreading worldwide, and it has been estimated that approximately 3 hectares of arable land are damaged by the accumulation of salt every minute (FAO 2006). Salinity can cause considerable damage to internal plant physiology and biological organs and can also result in biochemical abnormalities and metabolic problems due to the accumulation of reactive oxygen species (ROS) and subsequent damage to lipids, proteins and nucleic acids (Fridovich 1986; Wise & Nayler 1987; Imlay & Linn 1988). Several strategies have been proposed to overcome the problem of salinity stress and maximise plant production, namely using new plant breeds in addition to a mycorrhizal colonisation approach (Al-Karari *et al.* 2001; Cantrell & Linderman 2001; Asghari *et al.*, 2005). Although mycorrhizas have been intensively used in plant production and agricultural research, little is known about their ability to combat salinity (Giri *et al.*, 2002). Only a few studies have addressed the ability of mycorrhizas to mitigate soil salinity and improve plant production to date. For example, using mycorrhizal inoculant for the neem tree (*Azadirachta indica* A. Juss) over a range of different salinity levels increased dry matter biomass compared with non-mycorrhizal controls (Pande & Tarafdar 2002). Adding mycorrhizas to wild bean (*Strophostyles helvola* L.) increased the chlorophyll content and the amount of water in the plant, which in turn, made it more resistant to salinity (Tsang & Maun 1999). The main mechanism underlying the protective effect of mycorrhizas against soil salinity was suggested to be an increase in the absorption of phosphorus by the plant (Ojala *et al.*, 1983).

Different species of mycorrhiza can have different effects on the plant under salinity stress. For example, inoculation of wheat with different species of mycorrhizas under field salinity stress revealed that *Glomus etunicatum* had the greatest impact on plant productivity, followed by *G. intraradices* and *G. mosseae* (Daei *et al.*, 2008).

Another study on the effect of different species of mycorrhizas on olive trees under salinity stress showed that *G. mosseae* was the most effective species, followed by *G. intraradices* and *G. claroideum* (Porras-Sorriano *et al.*, 2009). Thus, the particular species of mycorrhiza and plant also plays an important role in determining the response of the plant under salinity stress. In addition, using single or mixed species of mycorrhizas can differently affect plant responses. As such, the theory of Functional Complementarity proposes that using multiple species of mycorrhizas rather than single species, adds more benefits to the plant growth behaviour and improves the resistance to different forms of environmental stress (Koide 2000).

The aim of this study was to determine the effect of different species of mycorrhizas (*G. etunicatum* and *G. mosseae*) individually and in combination on plants under salinity stress conditions. It was hypothesised that a mixture of mycorrhiza species, rather than individual fungi, confers better protection and stress resistance and enhances the plant ability to cope with salinity stress.

7.2 Methods

Plantago lanceolata (section 2.6.1) seeds were germinated in a controlled environment room (section 2.1). After reaching the four-leaf stage, the seedlings were transplanted into 11-cm square pots filled with commercial sterilised compost (section 2.1.3). The plants were inoculated with different mycorrhizal species either individually or in a mixed mycorrhizal inoculation. Plants were thus inoculated with *G. etunicatum*, *G. mosseae* and a mixed inoculation of both species, in addition to the control. The mycorrhizal-treated plants were maintained for two weeks before the salinity treatments were applied, to ensure that the mycorrhizas were established in the root system.

The experiments were divided into three salt treatments: 1.5 dS/m, 3.5 dS/m and 0 dS/m electrical conductivity (tap water) as the control (Appendix B). Each week, 100 mL of the salt solutions were applied to each of the plants in each treatment group to maintain the desired salinity level in the pot constant. Nutrient solutions were added to the plants at two-week intervals (as explained previously in section 2.1.4). The salt type used in this experiment was mixed salt.

The experiment lasted for four months in controlled room conditions (section 2.1) and at the end plant height, leaf number, inflorescence number and length were counted.

The weights of inflorescences were also measured, to indicate the weight of the seeds they contained. Initial shoot biomass and final dry shoot weight were taken for each plant, to calculate the final shoot biomass for each treatment (section 2.4). The roots for each plant were cleaned and stained for mycorrhizal visualisation (section 2.5.1). The stained roots were prepared on glass slides for AM fungal quantification and identification of different parts of mycorrhizas such as vesicles, hyphae and arbuscules (section 2.5.2).

The seeds produced from F1 plants in each treatment were used for the germination of F2 generation. From each plant, 15 healthy seeds were selected for the germination test. Petri dishes of 90 mm diameter with filter paper inside were used for the germination test at constant room temperature (26°C). The seeds of each plant were sub-divided into three Petri dishes, and five seeds were placed in each Petri dish, making a total of 15 seeds for each plant. The seeds were watered daily with distilled water and seedling germination was recorded for the seven days of the experiment, together with the final total shoot and root length for each successfully germinated seedling.

The experiment comprised six replicates per plants, three levels of salinity and four different treatments of mycorrhizas, which gave a 6 × 3 × 4 factorial for a total of 72 plants. All data were tested for normality, prior to factorial ANOVA employing salt and mycorrhizal species as the main effects with the Unistat version 6.0 statistical package.

7.3 Results

Plant height was reduced by salinity stress (Table 7.1). At the 3.5 dS/m salinity level, plant height decreased in comparison with that at lower salinity levels (Figure 7.1). With regard to the addition of different mycorrhiza species, *G. mosseae* enhanced plant height (Table 7.1). At 1.5 dS/m stress, *G. mosseae* caused an increase in plant height in comparison with other mycorrhizas (Figure 7.1); however, inoculation with *G. etunicatum* mycorrhizas did not affect plant height (Table 7.1).

Leaf number was significantly affected by salinity (Table 7.1); the leaf number at 1.5 dS/m was greater than that at the higher salinity level, as at 3.5 dS/m the number of leaves started to decrease (Figure 7.2). Inoculation with *G. mosseae* showed reduced

leaf production (Table 7.1). The final recorded leaf number was lower after the addition of *G. mosseae* at a low salinity level (Figure 7.2). However, *G. etunicatum* showed a significant interaction with salt, because fungal addition reduced the leaf number when no salt was added, but had no effect or a small positive effect when salt was present (Table 7.1; Figure 7.2).

Plant exposure to salinity considerably reduced final biomass production (Table 7.1). At the highest salinity level (3.5 dS/m), mean dry biomass was much lower than in the presence of lower salinity levels or no salt (Figure 7.3). Similar to leaf number, plant biomass showed an interaction between the addition of *G. etunicatum* and salinity stress (Table 7.1), as fungal addition reduced plant size when no salt was added, but had a small positive effect when salt was present in any concentration (Figure 7.3).

Table 7.1: Summary of the results of Analysis of Variance testing for the effect of different salinity stress levels and mycorrhizal inoculation on various vegetation parameters of *Plantago lanceolata*. Gm = *Glomus mosseae* and Ge = G. *etunicatum*. Degrees of freedom for salinity levels = 2,60; Gm = 1,60 and Ge = 1,60.

	Plant height (cm)		**Leaf number**		**Shoot dry biomass (g)**	
	F	**P**	**F**	**P**	**F**	**P**
Salinity	23.0	< 0.001	3.4	< 0.05	7.8	< 0.001
Gm	5.03	< 0.05	5.88	< 0.01	2.5	0.12
Ge	0.48	0.49	0.1	0.8	0.56	0.56
Salinity x Gm	1.9	0.15	1.9	0.15	0.21	0.81
Salinity x Ge	1.8	0.18	2.8	< 0.05	4.7	< 0.01
Gm x Ge	1.8	0.18	1.12	0.29	0.15	0.7
Salinity x Gm x Ge	0.65	0.53	1.3	0.29	0.27	0.77

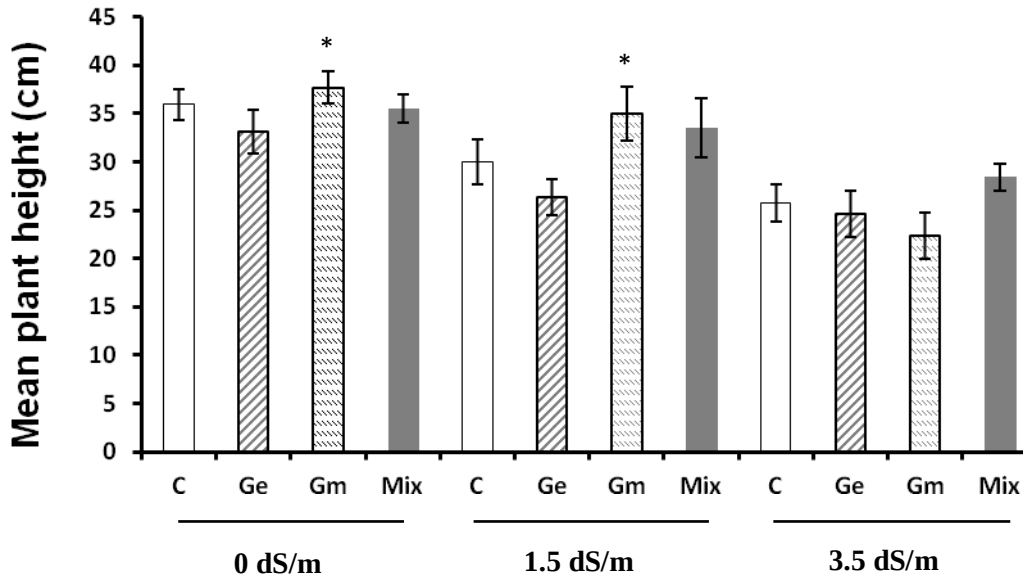

Figure 7.1: Mean plant height (cm) produced after inoculation with different mycorrhizal species under different salinity levels (dS/m). C (no mycorrhizas added), Ge (*Glomus etunicatum*), Gm (*Glomus mosseae*) and Mix (*G. etunicatum* + *G. mosseae*). An asterisk shows significant differences with respect to the AM effect within the groups.

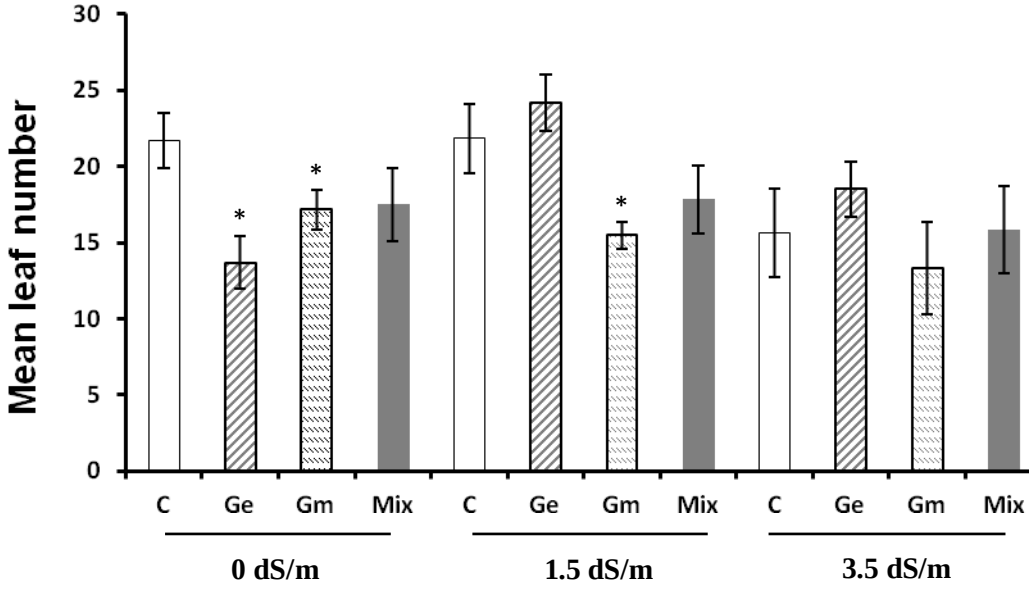

Figure 7.2: Mean plant leaf number produced under different salinity levels (dS/m) following inoculation with different mycorrhizal species. C (no mycorrhizas added), Ge (*Glomus etunicatum*), Gm (*G. mosseae*) and Mix (*G. etunicatum* + *G. mosseae*). An asterisk shows significant differences with respect to the AM effect within the groups.

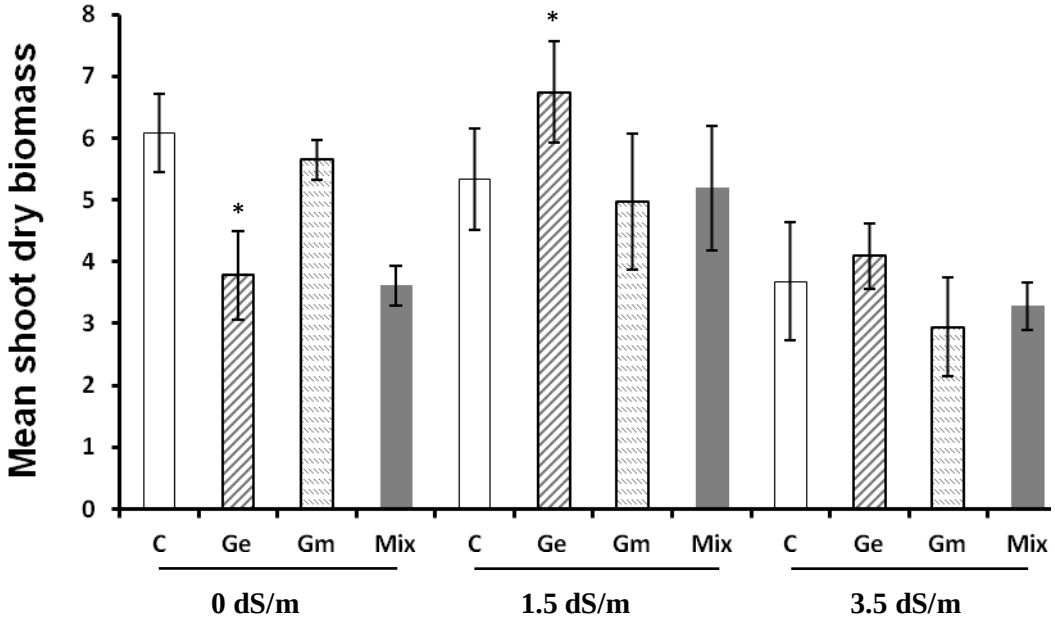

Figure 7.3: Mean plant final dry shoot biomass (g) after treatment with different salinity levels (dS/m) and mycorrhizas. C (no mycorrhizas added), Ge (*Glomus etunicatum*), Gm (*G. mosseae*) and Mix (*G. etunicatum* + *G. mosseae*). An asterisk shows significant differences with respect to the AM effect within the groups.

With respect to plant reproduction, salinity significantly increased the number of inflorescences produced by the plants under certain stress levels (Table 7.2). Exposure to the 1.5 dS/m level of salinity in particular resulted in a greater number of inflorescences than in the no salt or the higher salinity treatments (Figure 7.4). There was no overall effect of the addition of either mycorrhizal species (Table 7.2; Figure 7.4). However, addition of *G. etunicatum* increased flower production at the high level of salt and when no salt was added, but not at the low level of salt, leading to a significant interaction term (Table 7.2). Furthermore, although addition of either mycorrhiza increased flower production, no further increase was observed when both fungi were added, again leading to a significant interaction between the fungi (Table 7.2; Figure 7.4). A similar increase in inflorescence head length was observed in the presence of 1.5 dS/m salinity stress than in the absence of salinity or in higher salinity stress (Table 7.2; Figure 7.5). However, the combined addition of *G. mosseae* and *G. etunicatum* resulted in a sharp decrease in the head-length parameter in 0 dS/m and 1.5 dS/m saline treatments in comparison with the addition of either mycorrhizal fungus alone, producing a highly significant interaction term between the fungi (Figure 7.5).

Table 7.2: Summary of the results of Analysis of Variance testing for the effect of different salinity stress levels and mycorrhizal inoculation on the reproductive output of plants. Ge = *Glomus mosseae* and Gm = *G. etunicatum*. Degrees of freedom for salinity levels = 2,60; Gm = 1,60 and Ge = 1,60.

	Inflorescence number		Inflorescence head length (cm)	
	F	**P**	**F**	**P**
Salinity	5.5	< 0.001	4.01	< 0.05
Gm	0.1	0.75	0.34	0.56
Ge	1.6	0.21	0.98	0.33
Salinity x Gm	4.2	< 0.01	1.6	0.21
Salinity x Ge	1.2	0.32	1.5	0.24
Gm x Ge	4.3	< 0.05	8.5	< 0.001
Salinity x Gm x Ge	4.0	< 0.05	0.53	0.6

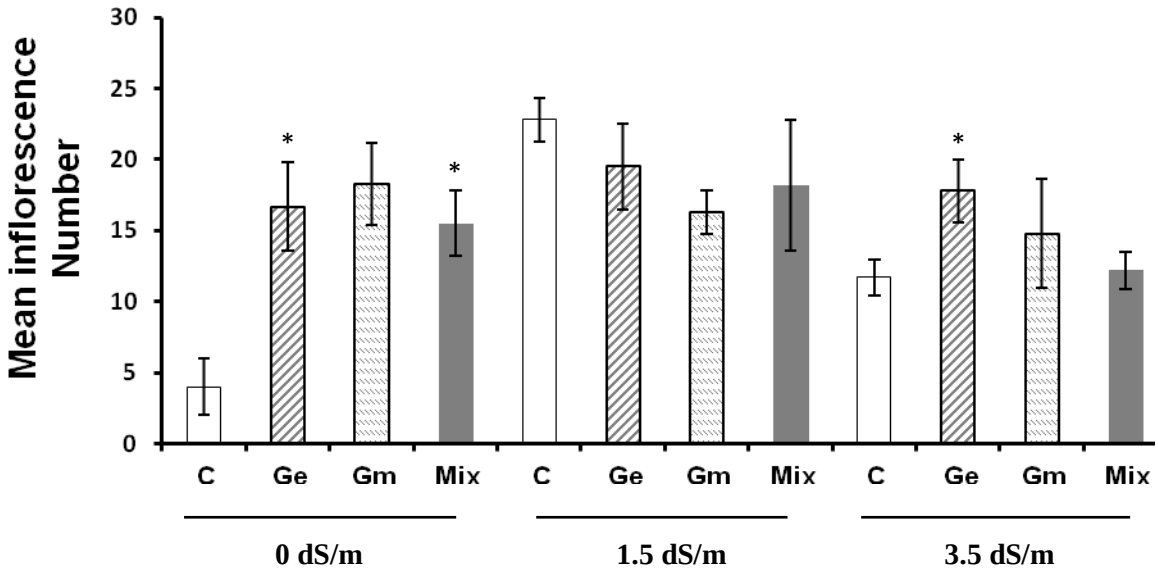

Figure 7.4: Mean number of inflorescences produced by plants under different salinity stresses (0, 1.5 and 3.5 dS/m) and different species of mycorrhizas. C (no mycorrhizas added), Ge (*Glomus etunicatum*), Gm (*Glomus mosseae*) and Mix (*G. etunicatum* + *G. mosseae*). An asterisk shows significant differences with respect to the AM effect within the groups.

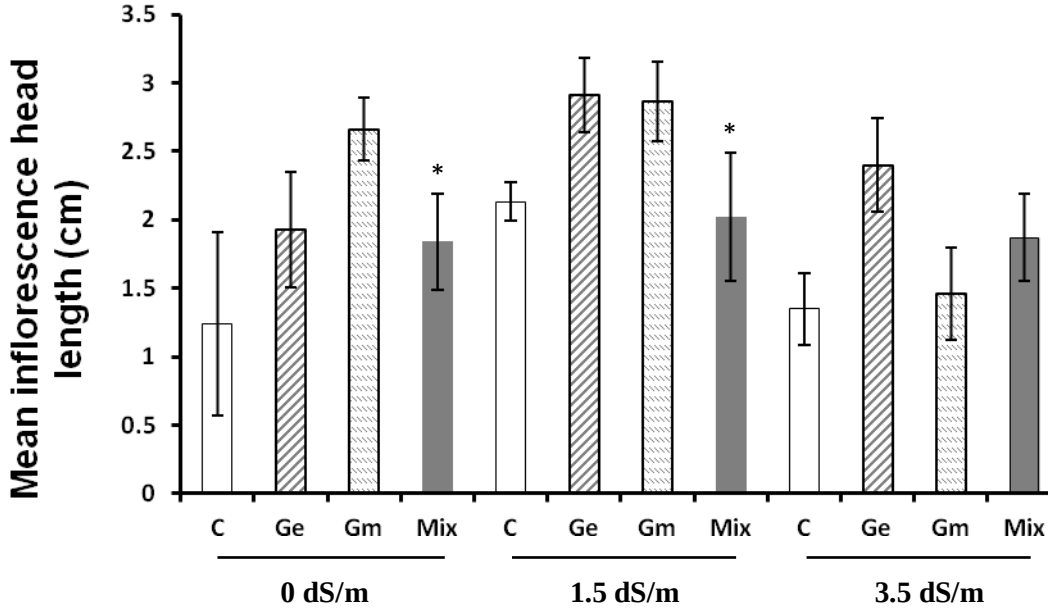

Figure 7.5: Mean inflorescence head length (cm) for *P. lanceolata* inoculated with different species of mycorrhizas under different salinity levels (0, 1.5 and 3.5 dS/m). C (no mycorrhizas added), Ge (*Glomus etunicatum*), Gm (*G. mosseae*) and Mix (*G. etunicatum* + *G. mosseae*). An asterisk shows significant differences with respect to the AM effect within the groups.

Salinity significantly enhanced the growth of second-generation seedlings (Table 7.3). Low salinity stress (1.5 dS/m) increased seed germination and seedling growth more than in the no-salt treatment or high salinity stress (Figures 7.6 and 7.7). The addition of *G. mosseae* had no overall effect on seedling performance, but a significant interaction was found between its addition and salinity (Table 7.3). *G. mosseae* tended to increase both seed germination and seedling growth in the absence of salt stress, but no effect on these variables was observed with salt stress and the effect of this fungus on seedling growth even appeared to be negative on occasions (Figures 7.6 and 7.7). Inoculation of parent plants with *G. etunicatum* had no effect on the growth rate and showed no interaction with salinity (Table 7.3).

Table 7.3: Summary of the results of Analysis of Variance testing for the effect of different salinity stress levels and mycorrhizal inoculation on second-generation plant offspring. Ge = *G. mosseae* and Gm = *G. etunicatum*. Degrees of freedom of salinity levels = 2,60; Gm = 1,60 and Ge = 1,60.

	Seed germination rate		Seedling length (cm)	
	F	**P**	**F**	**P**
Salinity	9.2	< 0.001	12.1	< 0.001
Gm	0.05	0.83	0.39	0.53
Ge	0.05	0.83	0.26	0.61
Salinity x Gm	4.03	< 0.05	3.4	< 0.05
Salinity x Ge	0.58	0.57	1.3	0.27
Gm x Ge	1.2	0.28	0.074	0.79
Salinity x Gm x Ge	0.45	0.64	0.78	0.46

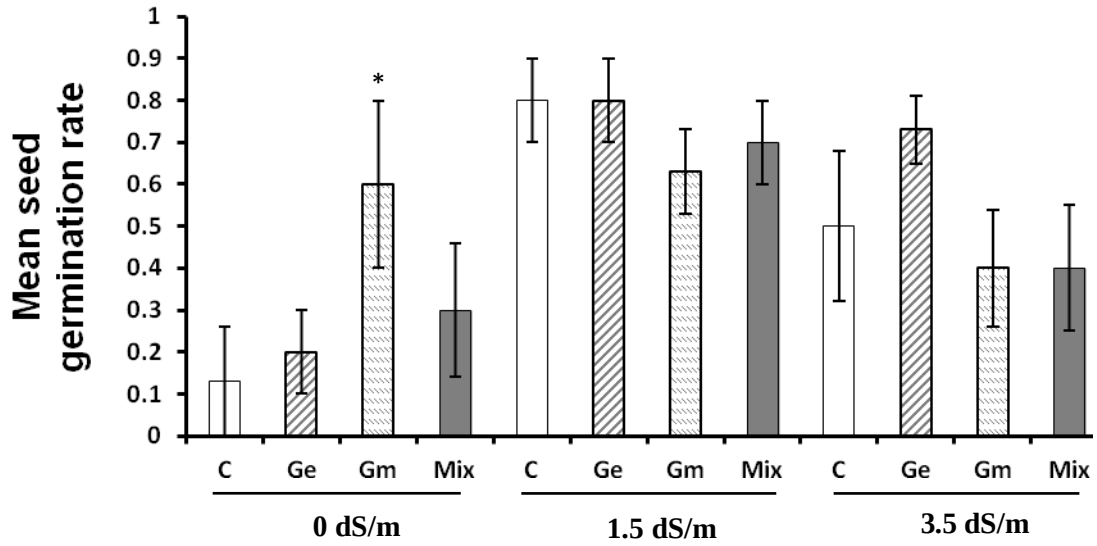

Figure 7.6: Mean offspring seed germination rate for *P. lanceolata* when the parental plants were inoculated with different species of mycorrhizas under different salinity levels (0, 1.5 and 3.5 dS/m). C (no mycorrhizas added), Ge (*Glomus etunicatum*), Gm (*G. mosseae*) and Mix (*G. etunicatum* + *G. mosseae*). An asterisk shows significant differences with respect to the AM effect within the groups.

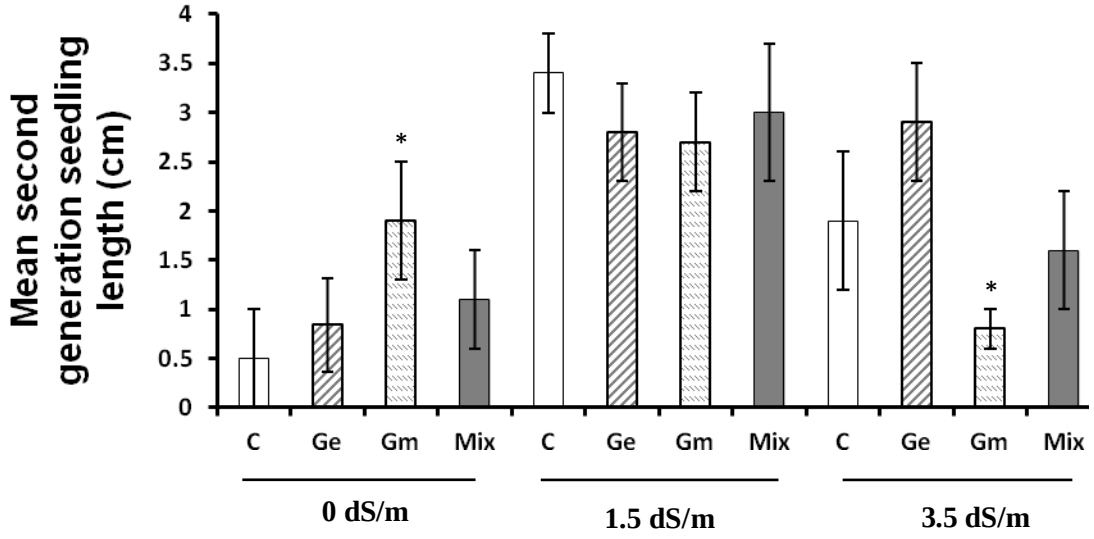

Figure 7.7: Mean offspring seedling length growth for *P. lanceolata* when the parental plants were inoculated with different species of mycorrhizas under different salinity levels (0, 1.5 and 3.5 dS/m). C (no mycorrhizas added), Ge (*Glomus etunicatum*), Gm (*G. mosseae*) and Mix (*G. etunicatum* + *G. mosseae*). An asterisk shows significant differences with respect to the AM effect within the groups.

Staining and visualisation of mycorrhizas in plant roots revealed different degrees of colonisation (Table 7.5). In the salinity control, *G. mosseae* showed the highest hyphal colonisation (30%) in comparison with other mycorrhizal inoculants. At the lowest salinity level (1.5 dS/m), the hyphal colonisation by *G. mosseae* and the mix was approximately double that of plants with no salt addition (Table 7.5). At the highest salinity level (3.5 dS/m), the colonisation rate of the hyphae was dramatically lower with all species of mycorrhizas used. Colonisation of hyphae by *G. etunicatum* was highest at 0 dS/m and decreased with increasing salinity levels. Colonisation by vesicles and arbuscules was generally low at all salinity levels (Table 7.5).

Table 7.4: The percentages of root mycorrhizal colonisation by different physiological parts. Treatments include salinity stress (0, 1.5 and 3.5 dS/m EC), and mycorrhizal species Ge (*Glomus etunicatum*), Gm (*G. mosseae*) and Mix (*G. etunicatum* + *G. mosseae*).

Treatments	Hyphae %	Vesicles %	Arbuscules %
0 EC + Gm	30	0	1
0 EC + Ge	25	0	0
0 EC + Mix	17	0	1
1.5 EC + Gm	42.5	2	3
1.5 EC + Ge	21	0	1
1.5 EC + Mix	28	1	1
3.5 EC + Gm	13	1	0
3.5 EC + Ge	9	0	0
3.5 EC + Mix	6	2	2

7.4 Discussion

The findings presented here indicate that the presence of mycorrhizas did not consistently enhance the growth of various plant parameters, regardless of the different salinity treatments applied in the study. Similar results were previously reported for *Lotus glaber* Mill., where the addition of mycorrhizas did not affect different growth parameters under salinity conditions (Echeverria *et al.*, 2008). Another example is the addition of *G. fasciculatum* to *Distichlis spicata*, which failed to enhance biomass and plant growth under salinity stress (Allen & Cunningham 1982). Here, the mycorrhizal species interacted differently under salinity stress and differentially influenced vegetation growth parameters. Notably, an enhancement of plant height and dry shoot biomass were observed with *G. mosseae* and *G. etunicatum*, respectively, under 1.5 dS/m salinity. It has been shown that adding mycorrhizas under salinity stress might not affect all aspects of vegetative growth positively, but might only enhance certain aspects of growth. The study of two salt marsh plant species using mixed species of mycorrhizas demonstrated only positive effects on the number of shoots and negligible benefits for all the other vegetative parts (McHugh & Dighton 2004).

In the context of reproductive organs (inflorescence number and head length), the data here showed that low salinity levels (1.5 dS/m) increased growth more than the

control and high salinity stress. Exposure to low salinity levels also increased shoot and root biomass and growth of carrizo citrange citrus in comparison with the absence of salt stress (Duke *et al.*, 1986). It has been suggested that in certain salinity conditions, different salt ions, especially sodium, act as enhancers of plant growth and suppress the negative effect of potassium (Mengel & Kirkby 1982). Thus, sodium at an application level of 1.5 dS/m appeared to act as an enhancer of plant growth instead of a suppressor in this study.

However, assessment of the impact of AM species on the reproductive physiology of *P. lanceolata* in the present investigation revealed a positive effect. Using mixed species of mycorrhizas caused plants to produce more inflorescences, whereas *G. etunicatum* significantly increased inflorescence number at 3.5 dS/m. Giri *et al.* (2003) studied the effects of mycorrhizas on *Acacia* trees in salinity situations and concluded that mixed species of AM significantly enhanced growth more than the use of individual *G. fasciculatum* and *G. macrocarpum* species. Further studies have revealed that using mixed species of mycorrhizas from different communities can provide more benefits to the plant than a single species alone, particularly with respect to plant height (Alkan *et al*., 2006; Koide 2000). In another study, mixed inocula of mycorrhizas increased the number of flowers and reduced the time to flowering (Gaur *et al.*, 2000). Nevertheless, different mycorrhiza species have distinct effects on growth and inflorescence number at different salinity levels; some mycorrhizas species enhanced the number of inflorescences produced at a low salinity level but had no effect in higher salinity treatments. Previous reports indicated that different AM fungi can affect plant growth variously at different salinity levels. At low EC salinity levels, *G. deserticola* enhanced shoot dry weight, but at a higher salinity level (1.5 dS/m), an unidentified *Glomus spp.* became effective (Ruiz-Lozano & Azcon 2000). The finding that the mixed addition of individual mycorrhizal species (*G. mosseae* and *G. etunicatum*) reduced the inflorescence head length (compared to that of plants exposed to either individual fungus) is consistent with previous reports that the addition of individual mycorrhizal species had a positive impact on vegetative growth (Ciftci *et al.*, 2010). Mycorrhizal colonisation gave different results for other vegetative parameters; the presence of multiple mycorrhizal species was not always associated with a positive outcome. For example, it has been suggested that the application of mixed species of *G. monosporus* and *G. tenus* might initiate a competition between AM species in the root system, which might in turn, reduce the beneficial outcome of the symbiosis with the plant (Wilson

1982). Hence, using mixed species of mycorrhizas will not necessarily be beneficial, as in some cases they can have a negative effect, as shown in the case of inflorescence head length in the current experiment. Overall, the results did not show a conclusive support for the theory of Functional Complementarity (Koide 2000), as in some situations using single species of mycorrhizas was associated with better outcomes.

The finding that inoculation of *G. mosseae* mycorrhizas positively affected seed germination and the growth of second-generation seedlings in comparison with inoculation by other mycorrhizal species was only observed in the absence of salinity stress. This agrees with the results from most other studies that confirmed the beneficial effects of mycorrhizal inoculation of parental plants on their offspring in certain conditions (Shumway & Koide 1994; Nuortila *et al.*, 2004; Varga 2009). Inoculation of *Glomus* species with *Campanula rotundifolia* L. produced seeds with a higher phosphorus content and faster germination rate (Nuortila *et al.*, 2004). However, there is again inconsistency in the literature, as certain mycorrhizal inocula can have a positive influence, whereas others show no response at all (Buwalda & Goh 1982). For example, mycorrhizal inoculation neither affected seed production in *Lycopersicon esculentum* Mill. plants (Bryla & Koide 1990), nor accelerated the germination rate of *Avena fatua* L. seeds, which displayed similar characteristics to the non-mycorrhizal treatment controls (Koide & Lu 1992). Some mycorrhizal species cannot improve plant growth and the supply of nutrients under salinity stress. It was previously demonstrated that salt ions can disturb the hyphal growth of mycorrhizas and inhibit carbohydrate supply to the plant (McMillen *et al*., 1997). Under salinity conditions, mycorrhizal hyphae require more energy to maintain the ionic balance in the mycelium (Cook & Whipps 1993), and it appears that the mycorrhizas used in this experiment failed to generate sufficient energy to overcome the stress and confer benefits to the plant. Thus, salinity can effectively influence seed germination, but the effect appears to be context-dependent (i.e., on species and situation).

As expected, mycorrhizal colonisation decreased as salinity stress increased. Similarly, using different species of mycorrhizas, Juniper and Abbott (2006) observed a decreased colonisation of roots with increasing salinity stress. Previous experiments on *Archaeospora trappei, Gigaspora decipiens* and *Scutellospora calospora* showed a reduction in root colonisation with an increasing addition of NaCl. However, the presence of 1.5 dS/m salinity increased hyphal colonisation by *G. mosseae* more than that of the control. A similar result was obtained with *Scutellospora calospora* (WUM

12-2), which showed an increased colonisation of plant roots at 13 dS/m salinity stress compared with the control (Juniper & Abbot 2006). Under certain environmental stresses such as cold, drought and darkness, mycorrhizal sporulation increased and provided maximum benefits for the plant to cope with the stress (Sylvia & Schenck 1983).

Overall, the data presented here indicate that the selected mycorrhizal species used in this experiment did not strongly enhance plant response to salinity stress and only improved certain parameters. This might be because the species of AM used were not from a saline habitat; according to previous studies, the habitat of origin of mycorrhizas is important in determining the outcome of any interactions with salt stress (Bentivenga *et al.*, 1997; Smith & Smith 1997; Bago *et al.*, 1998). It was shown that mycorrhizas originating from a saline habitat more effectively help plants tolerate salinity stress. The presence of mycorrhizas of saline origin reduced the Cl^- content of tomato leaves to help it tolerate soil salinity more than non-saline AM strains did (Copeman *et al.*, 1996). Furthermore, the presence of *G. mosseae* originating from a saline habitat helped cotton plants to tolerate high salinity stress and increased the concentration of phosphorus in plant tissues (Tian *et al.*, 2004).

7.5 Conclusion

The detrimental effect of salinity on various growth parameters was evident in the present study and the addition of AM fungal species improved some, but not all, growth parameters to alleviate the effects of salinity stress. However, the effect was context-dependent and many interactions were found between salt and mycorrhizal addition, which highlights the complexity of mycorrhizal effects on plant physiology. Although salinity levels of 1.5 dS/m increased some vegetative growth parameters in parents and offspring plants, the rate of growth decreased substantially with increased salinity stress, which might have masked any potential beneficial effects of fungal colonisation.

It is recommended that further research should be undertaken using individual or mixed AM species extracted from saline habitats, as mycorrhizas from saline-stressed environments are better adapted to salinity and tend to have different effects on plants under osmotic stress.

Chapter 8

A meta-analysis of different arbuscular mycorrhizal fungi-salinity experiments

8.1 Introduction

Soil salinity is associated with detrimental effects on the establishment, growth and development of plants leading to severe losses in agricultural yield (Giri *et al.*, 2003; Mathur *et al.*, 2007). The effects of salinity on plants are variable and may involve osmotic effects, specific-ion toxicity and/or nutritional disorders (reviewed in Evelin *et al.*, 2009). The devastating impacts of salinity are not only limited to the plant, but can also affect soil quality by reducing porosity and water permeability (Moghaieb *et al.*, 2004). Several methods have been implemented to fight soil salinity and increase agricultural productivity, namely using salt-tolerant plants, developing genetically engineered crops and extracting salts from irrigation water by mechanical means (Ashraf & Harris 2004; Flowers 2004; Zhang & Blumwald 2001). Despite the success, these approaches are expensive and financially unaffordable to poor farmers around the world (Cantrell & Linderman 2001).

According to many studies, the application of arbuscular mycorrhizal fungi can be an alternative cost-effective approach to overcome soil salinity stress via promoting salinity tolerance in plants. This involves several mechanisms, such as increasing plant growth by enhancing nutrient acquisition particularly phosphorus (Marschner & Dell 1994; Gerdemann 1975), defending the plant from many types of pathogens (Azcon–Agiar & Barea 1997), and helping the plant to overcome environmental stress during establishment (Grove & Malajczuk 1994). At the physiological level, mycorrhizas can increase gaseous exchange, transpiration rate and water use efficiency of plants under salt stress (Ruiz-Lozano *et al.*, 1996). They also increase root growth and spread to maximise nutrient absorption from the soil under salinity stress (Cantrell & Linderman 2001). Moreover, water absorption from saline soil is increased by the presence of the AM association due to improved osmotic adjustment in the cells of the root (Feng *et al.*, 2002). Even though the AM-plant association can be beneficial for the plant, multiple factors determine the outcome of this association, such as nutrient content of the soil,

soil moisture, soil pH and the interaction with soil bacteria and other microorganisms (Krikun 1991; Wilcox 1991; Fitter & Garbaye 1994).

In fact, not all of the AM associations with plants under salinity stress yield positive results. Several studies have shown that AM fungal associations are reduced by salt and do not enhance plant production (Gupta & Krishnamurthy 1996; Ruiz–Lozano & Azcon 2000). Also, mycorrhizas can be negatively affected by salinity through a reduction of their hyphal growth (Cantrell & Linderman 2001). Moreover, various species of AM fungi behave differently under salt stress due to differences in their genetic make-up (Tian *et al*., 2004). As a result of these factors, AM associations can give varying results in combating soil salinity. Here I utilised the meta-analysis method to integrate results from different experiments to gain insights into the plant response to mycorrhizas applied as single or mixed species inocula under salinity stress.

Meta-analysis is a statistical tool for quantitative data synthesis based on numerical analysis of data obtained from independent experiments addressing the same research question (Hedges & Olkin 1985; Koricheva *et al*., 2013). In meta-analysis, the outcome of each study is quantified and expressed as a common metric called an 'effect size' and the variance of this effect size can also be calculated in many studies (Gurevitch *et al*., 2000; Koricheva *et al*., 2013). Combining these effect size measures across studies is essential to estimate the grand mean effect size and its confidence interval, and to test whether this overall effect differs significantly from zero (Gurevitch *et al*., 2000; Koricheva *et al*., 2013). Meta–analysis methods have been used intensively in many scientific and social studies (Hedges & Olkin 1985), and have become popular in ecology and evolutionary biology as well (Arnqvist & Wooster 1995; Osenberg *et al*., 1999). With respect to AM studies, meta–analytic methods have been utilised to explore many different aspects of the fungal-plant relationship. For example, Treseder (2004) tested the AM-plant association with nutrient elements and CO_2 fertilisation, whilst Borowicz (2001) examined mycorrhizas and plant pathogen interactions. A further study used meta–analysis to compare the effects of different agriculture methods on AM colonisation of various crops (Lekberg & Koide 2005). The relative importance of the AM-plant symbiosis in relation to other types of interactions (Morris *et al.*, 2007) and the context-dependency in plant response to AM (Hoeksema *et al.*, 2010) were also investigated using meta-analytic methods.

In the present study, a meta-analysis was conducted to integrate and compare the results of different published experiments on salinity stress and the diverse effects of AM fungi. The aim of the present study was to generally analyse the magnitude of the effect of AM fungi inoculation on plant growth and establishment under salt stress through a quantitative meta-analytic approach. The analysis was conducted using data that have salt added, with or without mycorrhizas supplemented as a single inoculum or mixed species, in order to determine if the fungal-plant association can mitigate the effects of salt addition. I hypothesised that mycorrhizal application positively affects plant response under salinity stress. The mycorrhizal effects were evaluated in the context of different environmental conditions (field and controlled) and various experimental factors (salt type, salinity levels and bacterial addition) across the independent studies.

8.2 Methods

8.2.1 Literature search and data collection

The search engines used for the selection of the relevant scientific publications were Google Scholar and ISI Web of Science®. The search on Google Scholar used the following words: "Mycorrhizas + Salinity". With respect to the ISI search, the search combinations entered were "Mycorrhiza* + Salinity", "mycorrhiza* inoculation and salt stress/or under salinity stress", "Arbuscular Mycorrhiza* inoculation and salinity stress/or under salt stress". Using the "*" character throughout the search on ISI ensured that only papers containing mycorrhizal research were selected and that words such as mycorrhizae, mycorrhizas and mycorrhizal were also included. Published articles were selected for this study that had different treatment means, sample sizes and standard deviations for both the controls and the parameters being investigated (Gurevitch *et al*., 1992). For scientific papers that did not include the standard error or the standard deviation, the authors were contacted by email to clarify the lack of data. Unfortunately none of the first authors responded to the request to clarify the standard deviation and hence these studies were not included in the analysis. As a result, journals from 1980s until the recently published ones were searched. Scientific journals that were examined include: *African Journal of Agricultural Research; Agricultural Sciences in China;*

Applied Microbiology; Applied Soil Ecology; Biology and Fertility of Soils and Microbial Ecology; Biologia Plantarum; Brazilian Society of Plant Protection; Canadian Journal of Microbiology, Agriculture, Ecosystems & Environment; Colloids and Surfaces; Crop Protection ; Horticulture Science; International Journal of Plant Protection; Journal of Japan Horticulture Science; Journal of Plant Growth Regulation; Microbial Research; Mycorrhiza; New Phytologist; Pius Floris Special Report; Plant and Soil; Plant Physiology and Biochemistry. Journal articles included in the current study are listed in Table 8.1.

8.2.2 Data management

Data from the different articles were recorded in an Excel spreadsheet. Different sets of information were recorded and categorised for comparison, such as: plant species, plant family, experimental conditions, salt types, salinity levels etc. In addition, columns were created to represent response variables for different parameters and were categorised as vegetation and chemical elements. The parameters in the vegetation category included quantities such as shoot dry weight, leaf number, shoot height etc. Elements were included in the chemical category, such as nitrogen, phosphate, sodium etc.

8.2.3 Meta-analysis

The meta-analysis was carried out by using MetaWin Version 2.0 statistical program (Sinauer Associates, Sunderland, MA), a comprehensive package for performing modern meta-analysis (Rosenberg *et al*., 2000). Basically, the meta-analysis consists of two main stages: (1) calculating individual effect sizes and their associated variances from each study in order to place the data on a common scale; (2) combining these effect sizes in a statistical summary based on a particular meta-analytic model (Rosenberg *et al*., 2000). Several methods are available to calculate effect sizes, such as the standardised mean difference (Hedges 1981; Hedges & Olkin 1985) and response ratio (Hedges *et al*., 1999). To measure the effect size in the present study I used the common meta-analysis metric of standardised effect size, Hedges's *d*, an unbiased modified form of Hedges's *g* (Hedges 1981) created by multiplying the latter with a correction factor (Hedges & Olkin 1985; Gurevitch & Hedges 2001). Hedges's *d* represents the standardised mean difference between treatment and control means

divided by the pooled standard deviation, and multiplied by a correction factor to account for small sample size bias (Hedges & Olkin 1985; Gurevitch *et al*., 2000). The statistical analysis (conducted by MetaWin program) was based on the grand mean effect size (E_+) estimation representing the overall magnitude of effect combined from the studies. Briefly, standardised individual effect sizes '*d*' are calculated by Hedges's equation (Appendix C). Once all effect sizes of the individual studies are acquired, the overall pooled mean effect size estimate 'E_+' is calculated by utilising a statistical computing software program (MetaWin), using direct weights defined as the inverse of the variance of '*d*' for each study, and providing a confidence interval for 'E_+' with a chi-square statistic and with the probability of this pooled effect size being equal to zero (Hedges & Olkin, 1985) (see Appendix C for further details). It is essential to see if the mean effect size for each group is significantly different from zero. Estimates of the effect size of the treatment group were considered to be significantly different from the control if the overall effect size and the 95% confidence interval (CI) around the effect do not overlap zero (Gurevitch *et al*., 2000; Rosenberg *et al*., 2000). Accordingly, if the mean difference between the AM inoculated (treatment) and the non-inoculated (control) group intersects zero, then there is no significant difference between the groups (i.e. no improvement with AM treatment); a positive effect size indicates a positive effect of mycorrhizas on plant growth under salinity, whereas a negative value indicates worsening of plant response with AM inoculation (as described in Gurevitch *et al*., 2000; Rosenberg *et al*., 2000). In general, the magnitude of overall effect sizes is commonly interpreted as small (≥0.2), medium (≥0.5), large (≥0.8), and very large (≥1) (Gurevitch & Hedges 1993). Difference between subgroups (i.e. single vs. mixed AM species) was inferred from whether the 95% CIs around the effect sizes were overlapped with each other; if both CIs were not overlapping, it is suggested that the difference was significant between both subgroups (Gurevitch *et al*., 2000; Koricheva *et al*., 2013). The analyses were conducted separately for the different variables (i.e. field condition, controlled conditions, salt type, salinity levels and bacterial addition) but the related factors were presented on the same graph for easier interpretation, as done in Gurevitch *et al.* (2001) and Hoeksema *et al.* (2010).

Table 8.1: Articles used in the meta–analysis, with the associated host plant common and Latin names, and whether AM fungi were used as a single or mixed source of inoculum.

Article	Plant name	Mycorrhizas
Allen & Cunningham (1983)	Saltgrass (*Distichlis spicata* L.)	Single
Duke *et al*. (1986)	Citrange (*Poncirus trifoliata* [L.] Raf. ×Citrus sinensis [L.] Osbeck)	Single
Hatimi (1999)	Golden wreath (*Acacia saligna* (Labill.))	Mix
Ruiz–Lozano & Azcon (2000)	Lettuce (*Lactuca sativa* L.)	Single
Cantrell & Linderman (2001)	Lettuce (*Lactuca sativa* L.)	Mix
Weissenhorn (2002)	Horse chestnut tree (*Aesculus hippocastanum* L.)	Single
Yano-Melo *et al*. (2003)	Banana (*Musa* sp. cv. Pacovan)	Single
Ghorbanli *et al*. (2004)	Soybean (*Glycine max* (L.) Merr cv.Pershing)	Single
Giri & Mukerji (2004)	Hummingbird tree (*Sesbania grandiflora* (Pers.))	Single
Tian *et al*. (2004)	Cotton (*Gossypium arboreum* L.)	Single
Ashgari *et al*. (2005)	Saltbush (*Atriplex nummularia* Lindl.)	Mix
Kashyab & Sharma (2005)	Wild mint (*Mentha arvensis*)	Mix
Hajiboland *et al*. (2006)	Tomato (*Solanum lycopersicum* L.)	Single
Kashyap & Sharma (2006)	White mulberry (*Morus alba* L.)	Mix
Muok & Takaaki (2006)	Marula (*Spondias birrea* A. Rich.)	Single
Oliveira *et al*. (2006)	Grey willow (*Salix atrocinerea* Brot.)	Single - Mix
Sannazaro *et al*. (2006)	Narrow leaf bird (*Lotus glaber* (Mill.))	Single
Aroca *et al*. (2007)	Common bean (*Phaseolus vulgaris* L.)	Single
Giri *et al*. (2007)	Arabic gum tree (*Acacia Senegal* L.)	Single
He *et al*. (2007)	Tomato (*Solanum lycopersicum* L.)	Single
Jahromi *et al*. (2007)	Lettuce (*Lactuca sativa* L.)	Single
Ashgari (2008)	Subterranean clover (*Trifolium subterrane*um L.)	Single

Article	Plant name	Mycorrhizas
Echeverria *et al*. (2008)	Foot trefoil (*Lotus corniculatus* L.)	Single
Garg & Manchanda (2008)	Pigeon pea (*Cajanus cajan* (L.) Millsp.)	Single
Sheng *et al.* (2008)	Maize (*Zea mays* L.)	Single
Shcng *et al*. (2009)	Maize (*Zea mays* L.)	Single
Gamalero *et al*. (2010)	Cucumber (*Cucumis sativus* L.)	Single
Huang *et al*. (2010)	Tomato (*Solanum lycopersicum* L.)	Single
Scambato *et al*. (2010)	Argentine mesquite (*Prosopis alba*)	Single
Borde *et al*. (2011)	Bajra (*Pennisetum glaucum* Jester)	Single
Evelin *et al*. (2011)	Fenugreek (*Trigonella foenum-graecum*)	Single
Garg & Aggarwal (2011)	Pigeon pea (*Cajanus cajan* (L.) Millsp.)	Single
Peng *et al*. (2011)	Chinese milkvetch (*Astragalus membranaceus*)	Single
Sheng *et al.* (2011)	Maize (*Zea mays* L.)	Single

(Table 8.1 continued)

8.3 Results

The first meta–analysis results were addressing the effect of AM inoculation in salinity experiments on host plants under different environmental conditions. Effect size refers to the AM inoculation effect on plant overall response under salinity stress as compared to the non-inoculated control. In the field experiments, the overall effect size of AM inoculation as a single inoculum in saline soil was positive and significantly different from the non-mycorrhizal controls according to the 95% CI limits (E_+ = 1.36, CI = 1.11 to 1.63; Figure 8.1). The multiple AM species inoculation also showed a positive enhancement of the plant response under salinity stress across studies (E_+ = 2.48, CI = 1.96 to 3.24; Figure 8.1).

In the controlled environment condition, mycorrhizal colonisation had a significant positive effect on plant establishment in saline soils, as evidenced by the positive overall effect sizes and the CIs that do not overlap zero, with both the single and multiple species inoculation (E_+ = 0.71, CI= 0.61 to 0.82; E_+ = 1.44, CI= 1.21 to 1.69, respectively; Figure 8.1). The mycorrhizal effects integrated from the experiments performed in the field and controlled settings (with the exception of the single inoculum in the controlled condition) can be described as very large effects (≥1) according to the Gurevitch and Hedges (1993) reference scale. Furthermore, the results revealed that using mixed AM species offered significantly larger benefits to salt-stressed plants compared to the single inoculation (Figure 8.1).

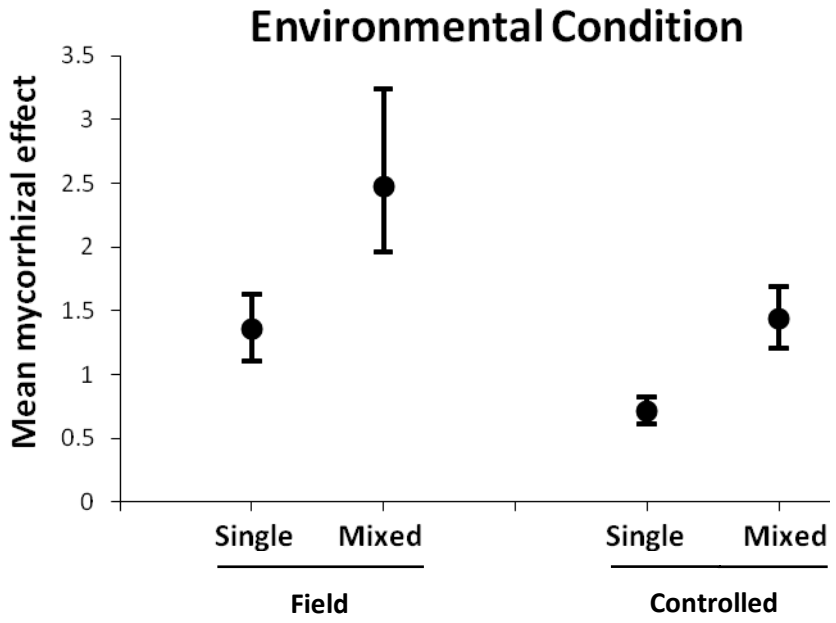

Figure 8.1: Effect size (mean ± 95% confidence interval) of arbuscular mycorrhizal inoculation in salinity studies under different environmental conditions (results are shown separately for field and controlled environment).

With respect to salt type, experiments employing multiple salts showed that single mycorrhizal inoculation had a medium positive effect on the host plants during salinity stress (E_+ = 0.61, CI = 0.37 to 0.85; Figure 8.2), while the multiple AM species addition produced no difference compared to the control (i.e. mean effect size intersects zero; Figure 8.2). On the other hand, a very large effect (E_+ = 1.53, CI= 1.3 to 1.78; Figure 8.2) was obtained from the mixed mycorrhizal species treatment under the NaCl salt addition. With the same salt type, the effect of single AM treatment was large positive (E_+ = 0.81, CI= 0.71 to 0.92; Figure 8.2) but significantly less efficient than the mixed species effect (Figure 8.2).

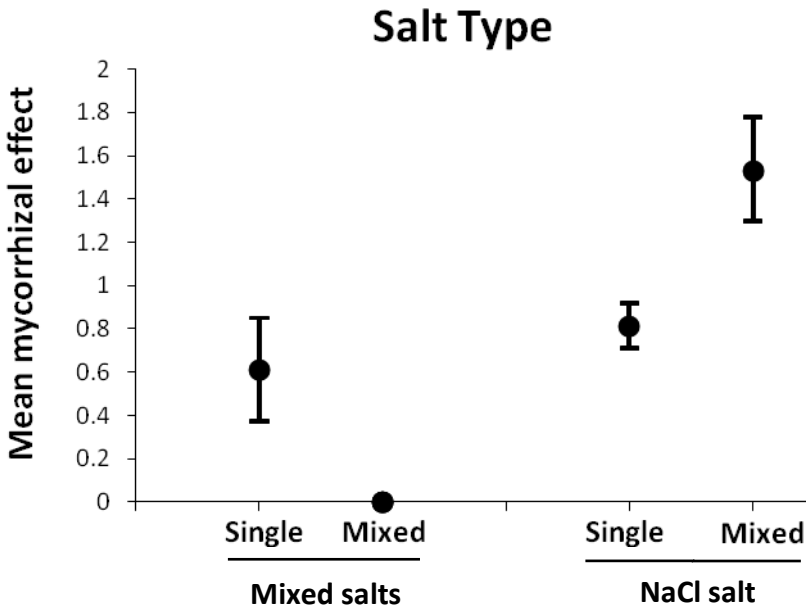

Figure 8.2: Effect size (mean ± 95% confidence interval) of arbuscular mycorrhizal inoculation (as single or mixed species) across salinity experiments using mixed salts or NaCl (results for each salt type are shown separately).

Examining the mycorrhizal effect under different levels of salinity showed a consistent positive improvement of growth parameters in salt-stressed plants under all salinity exposures (Figure 8.3). The overall effect sizes of AM inoculation under the different salinity levels were positive and significantly different from zero (Figure 8.3). At a low salt concentration (EC ranging from 1 to 4 dS/m across studies), the effects of single and multiple AM species were almost similar, with respective mean effect size values and 95% CIs as follows: E_+ = 0.72, CI = 0.59 to 0.85, and E_+ = 0.43, CI = 0.12 to 0.91 (Figure 8.3). A similar trend was observed under high salinity (≥ 8 dS/m) level (E_+ = 0.9, CI = 0.64 to 1.16 for single species, and E_+ = 0.9, CI = 0.59 to 1.25 for mixed species; Figure 3.8). Strikingly, at the medium salinity (4-8 dS/m) level the addition of mixed AM fungi to the plant was significantly more effective than the single AM inoculum in alleviating salinity stress on host plants as manifested by the very large mean effect size (E_+ = 1.89, CI = 1.59 to 2.19 for mixed species c.f. E_+ = 0.81, CI = 0.68 to 0.95 for single species; Figure 8.3).

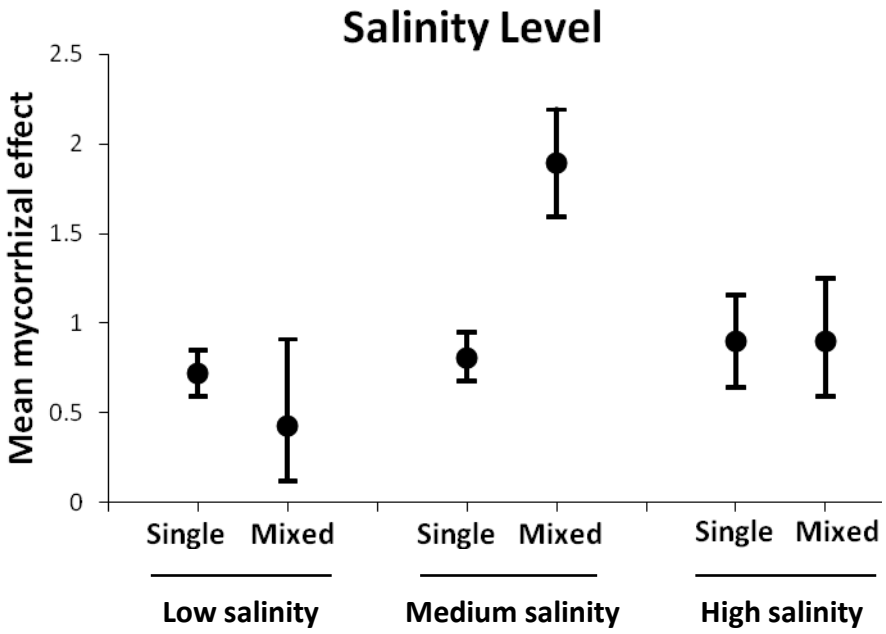

Figure 8.3: Effect size (mean ± 95% confidence interval) of using single or mixed arbuscular mycorrhizal fungal species under three levels of salinity (low, medium and high) across many experiments. Results for each salinity level are shown separately.

Another variable that was analysed across the studies in the meta-analysis was the bacterial addition in the presence of mycorrhizas and salt to gather evidence from the studies on whether AM fungi can alleviate the detrimental effects of salinity when bacteria is concomitantly present. Using soil bacteria in addition to AM fungi to resist soil salinity showed a significant positive response (E_+ = 0.8, CI = 0.63 to 0.97 for single AM; and E_+ = 2.22, CI = 1.77 to 2.72 for mixed AM; Figure 8.4). Interestingly, bacteria seemed to work significantly better with multiple AM species than with a single AM species inoculum (Figure 8.4).

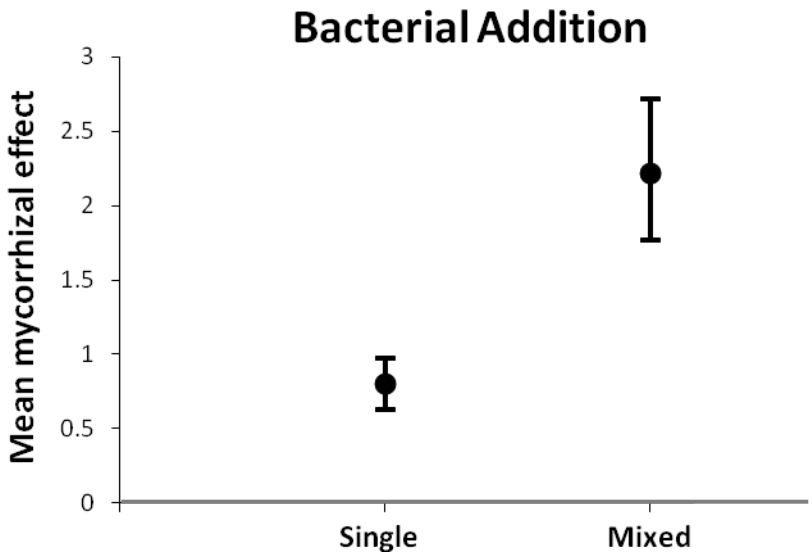

Figure 8.4: Effect size (mean ± 95% confidence interval) of arbuscular mycorrhizal inoculation (as single or mixed species) with bacteria under salinity stress.

Lastly, integrating the results of vegetation parameters only, such as shoot dry weight, leaf number, shoot height etc., across all the studies irrespective of any other experimental factors, also confirmed the positive impact of AM colonisation in mitigating salinity stress, as evident by the very large mean effect size values in the positive direction (Figure 8.5). Moreover, the vegetation results revealed that using a mix of AM species was superior to the single species in enhancing plant growth parameters under salinity stress (E_+ = 1.56, CI = 1.31 to 1.81 for mixed species c.f. E_+ = 0.96, CI = 0.79 to 1.12 for single species; Figure 8.5).

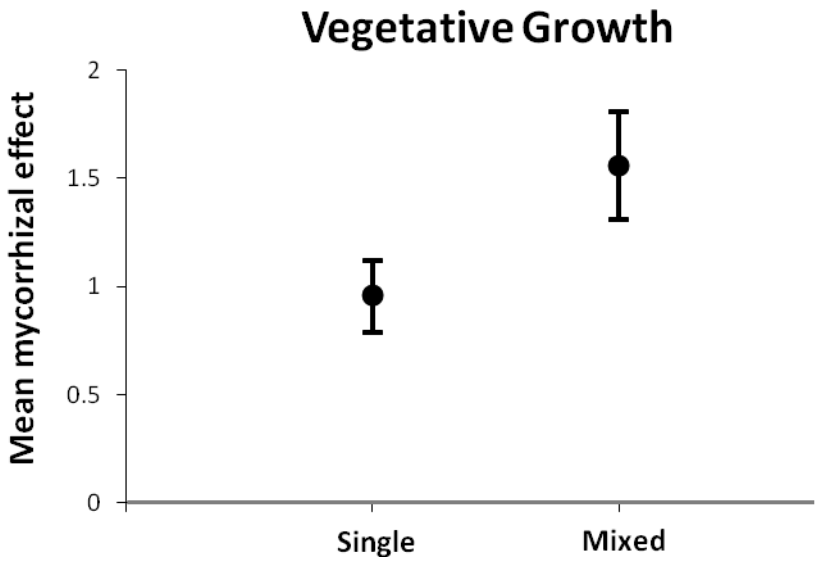

Figure 8.5: Effect size (mean ± 95% confidence interval) of arbuscular mycorrhizal inoculation (as single or mixed species) on vegetation parameters across experiments using different levels of salinity stress.

8.4 Discussion

Using the meta-analysis technique, the results of several trials from 34 published articles were integrated to ask whether AM fungal inoculation can mitigate salt stress, and whether the complexity of the fungal inoculum (i.e. single vs. multiple species) would affect the plant differently under salinity stress. The analysis showed a remarkably significant and consistent enhancement of plant responses with AM treatment under various conditions.

The current meta-analysis demonstrated that mycorrhizal symbiosis significantly mitigated salinity stress in natural field systems as well as in controlled experimental conditions. Moreover, the results confirmed that both the single and multiple AM inoculation enhanced plant performance at all salinity levels, with different salt types and in the presence of bacteria. A considerable body of evidence advocates that colonisation with single species of mycorrhizas is effective in enhancing plant ability to overcome salt stress (Aroca *et al*., 2006; Sannazzaro *et al*., 2007; Zhi *et al*., 2010). Also, previous experiments showed that using multiple species of mycorrhizas have a positive overall impact on plant under salinity stress (Giri *et al*., 2003; Peng *et al*., 2010; Zhang *et al*., 2011). It has been reported that AM inoculation reduces salinity-induced growth suppression and improves plant biomass under saline conditions (e.g., Tian *et al*., 2004;

Sannazzarro *et al.*, 2007; Garg & Manchanda 2009; Kaya *et al.*, 2009; Estrada *et al.*, 2013). There are different ways by which mycorrhizas can help plants to overcome salinity stress. As such, mycorrhiza has been shown to enhance the absorption of poor mobility nutrients in the soil, especially phosphorus (Al Karaki & Clark 1998). Additional evidence points toward the ability of mycorrhizas to attenuate salinity-associated damage by triggering the production of antioxidants (Sharifi *et al.*, 2007), increasing photosynthesis (Sheng *et al.*, 2008), maintaining ionic balance (Zuccarini & Okurowska 2008), improving water use efficiency (Dell'Amico *et al.*, 2002), reducing osmotic potential of plant cell, and increasing water uptake (Colla *et al.*, 2008).

For each condition examined, using multiple AM species seemed to confer more benefits to the salt-stressed plants, except when mixed salt type was used and under low and high salinity levels, which did not show the same trend. Many studies have confirmed that AM species respond differently according to the environmental condition of the experiments (McGonigle 1988; Newsham *et al*., 1995; Pringle & Bever 2008). Giri *et al*. (2003) used two species of AM fungi, *G. fasiculatum* and *G. macrocarpum*, both singly and in combination, and showed that under controlled environment conditions the AM combination was better able to increase *Acacia auriculiformis* Benth. plant resistance to salinity stress. Another field study concluded that using a mixture of AM species is important in establishing certain plant communities (Van der Heijden *et al*., 1998). Thus, throughout the majority of the experiments, either under controlled or field conditions, a mix of AM species was shown to enhance plant growth more than when using a single AM species, a finding that is consistent with Koide's Functional Complementarity theory (Koide 2000). The experiments conducted in previous chapters of this thesis revealed an improvement of some plant parameters with the commercial AM fungi under salinity stress in the field (Chapter 5) and glasshouse (Chapter 6) conditions, and a lack of colonisation in the controlled room environment with the commercial AM mix (Chapter 4 and Chapter 6) but not with the single *G. mosseae* and *G. etunicatum* species (Chapter 7). As discussed previously, the problem of unsuccessful AM-plant association could be attributed to several factors, namely unfavourable conditions in the CER, effect of soil pH (Clark 1997), impact of heavy metals (Marschner 1991), release of chemical inhibitors (Vierheiling & Ocambo 1990), effect of temperature (Heinemeyer & Fitter 2003) and insufficient lighting of the room (Aguirrezabal *et al*., 1994).

Although the meta-analysis generally illuminated the positive role of mycorrhizas in alleviating salt stress across diverse studies, the findings in previous chapters showed a degree of context-dependency of mycorrhizal effects in *P. lanceolata*. As such, results from the field experiment in Chapter 5 showed an improvement of some parameters with the commercial mycorrhizal mix under 2.2 dS/m salinity level, namely increased leaf number and inflorescence number. Moreover, the commercial mix of AM fungi significantly increased plant height and leaf number at 3.5 dS/m and 1.5 dS/m, respectively, in the glasshouse experiment (Chapter 6). In Chapter 7, inoculation with single AM species produced a positive effect on plant height with *G. mosseae* (at 1.5 dS/m) and increased dry shoot biomass (at 1.5 dS/m) and inflorescence number (at 3.5 dS/m) with *G. etunicatum*. Strikingly, these effects were lost with the mixed *G. mosseae* and *G. etunicatum* inoculation, a finding that can be explained by the possible competition between these AM species in the root system as suggested by Wilson (1982). The inconsistency between the results from the mixed *G. mosseae* and *G. etunicatum* inoculation and the meta-analysis finding might be essentially due to the differences in the AM species as well as plant families included in the meta-analysis which covered a wide range of AM fungi and plants across different studies and was not limited only to *G. mosseae* and *G. etunicatum* nor including *Plantago*. In fact, it is well known that plant responses under salinity stress vary with different AM species. For instance, inoculation of wheat with different AM species under field salinity stress showed improved outcomes with *G. etunicatum*, followed by *G. intraradices* and *G. mosseae* (Daei *et al*., 2008), whereas other reports suggested *G. fasciculatum* to be the most efficient in alleviating salinity stress in *Acacia nilotica* (Giri *at al*., 2007). Hence, the particular species of mycorrhizas and plants is critical in determining the plant response under salinity.

Different types of salt have different effects on mycorrhizas during stress. Results showed that the effectiveness of mixed AM species in the majority of the meta-analysed studies was negated with mixed salts, but that the opposite occurred when only NaCl salt was added. In agreement, the commercial AM mix used in Chapter 6 failed to associate and produce any positive effect under salinity stress induced by mixed salts in the controlled environment condition. Also, the combined inoculation with *G. mosseae* and *G. etunicatum* in Chapter 7 did not significantly enhance plant responses under mixed salt-provoked salinity stress. However, in the field experiment (Chapter 5) increased leaf number was observed in AM-inoculated plants with the mixed salt and

not with NaCl, reflecting the variability of response to different salts under different experimental settings. In this regard, a previous study by Fuzy *et al*. (2008) showed that *G. geosporum* improved plant resistance under a mix of salts in a marsh area, but failed to show any resistance when the experiment was switched to single NaCl salt addition. Several studies have also reported the detrimental effect of NaCl on individual AM fungal species (Giri *et al*., 2007; Juniper & Abbott 2006; Sheng *et al*., 2008). Despite the documented negative effects of salinity on mycorrhizal spore germination and root colonisation (Juniper & Abbott 2006; Evelin *et al.*, 2009), many reports indicate that mycorrhizas can still be symbiotically effective under saline conditions depending on the identity of the AM species (Giri & Mukerji 2004; Daei *et al.*, 2009; Evelin *et al.*, 2012; Talaat & Shawky 2014). Multi-factorial meta-analyses conducted by Hoeksema and colleagues (2010) proved that the identity and diversity of mycorrhizal fungi as well as the host plant identity are critical factors in determining the variation in plant response to mycorrhizal inoculation. These authors also suggested that the experimental site (laboratory vs. field) *per se* is a relatively less important factor than the plant and fungal characteristics in the symbiosis (Hoeksema *et al*., 2010). Meanwhile, another meta-analysis emphasized, in sharp contrast, the importance of the location and showed a greater plant response to AM in the glasshouse than in the field (Lekberg & Koide 2005). This might explain the improved outcome with the commercial AM fungal mix observed in the glasshouse experiment in Chapter 6, which should be taken into consideration for future experiments.

The current meta-analysis also revealed that across studies, different levels of salinity gave diverse responses with mixed or single mycorrhizal species. The effects of single and multiple mycorrhizas on plant performance did not differ under low (1-4 dS/m) and high (≥8 dS/m) salinity conditions, but more positive effects were seen with mixed species at the medium (4-8 dS/m) salinity level. In this regard, Trujilo (2006) confirmed that AM fungi performed better at low and medium salinity levels, but at high soil salinity stress mycorrhizal effects on plants were weakened. However, only few studies showed a persistence of mycorrhizal activity at high soil salinity (Aliasgharzadeh *et al*., 2001; Landwehr *et al*., 2002). It has been previously reported that at a high salinity level, the ability of AM fungi to absorb phosphorus from the surrounding soil declines sharply, thereby making AM fungi less helpful for the plant to overcome soil salinity (Shokri & Maadi 2009). Phosphorus is important as it increases the growth rate of the plant under stress through enhancing antioxidant production and

elevating nitrogen fixation in some plant species (Feng *et al*., 2002; Alguacil *et al*., 2003; Garg & Manchanda 2008). Linking this meta-analysis result to the findings from previous chapters revealed that the reported significant changes in AM-colonised plants occurred at salinity levels below 4 dS/m (specifically at 1.5, 2.2 and 3.5 dS/m), which is supported by the meta-analysis result at the low salinity level only. Unlike the meta-analysis result, the mycorrhizal effects were lost with higher salinity levels (5 and 10 dS/m; Chapter 5) possibly due to the detrimental effect of salinity on AM colonisation (Kaya *et al*., 2009), limiting spore germination (Hirrel 1981) and hyphal spread (McMillan *et al*., 1998) in the soil. Thus, high salinity levels were not used in further experiments.

Using soil bacteria has been shown to enhance the effect of the AM fungal association (Hoeksema *et al*., 2009). Some soil bacteria help mycorrhizas to establish in the soil and to increase colonisation of the host plant. For this reason they are called 'mycorrhiza helper bacteria MHB' (Garbaye 2006). Here I show that using AM fungal species together with the addition of bacteria in the soil gave a positive effect on plant response under salinity. This is supported by a previous study showing improved arbuscular mycorrhizal fungal functionality with bacteria (Hart & Reader 2002). Also, it was confirmed that a more diverse soil microbial community has positive effects on plant establishment and ecosystem health (Brooker *et al*., 2008; Harris 2009).

The majority of the studies analysed herein have confirmed the significant benefits of mycorrhizal colonisation in enhancing plant vegetation growth parameters under salt stress. Findings from the previous chapters agreed in some, but not all, situations with the meta-analysis results as AM colonisation increased leaf number, plant height, dry shoot biomass and inflorescence number under certain salinity levels only (see Chapters 5 and 6 for details). This variability in outcome is not surprising as the meta-analysis combine different studies conducted under various experimental conditions that are not necessarily identical to our experiments. Additionally, the plant species (Table 8.1) and the AM fungal groups from the studies included in the meta-analysis are diverse and not limited to *Plantago lanceolata* or the commercial AM mix, *G. mosseae* and *G. etunicatum* that were used in the previous experiments.

Plant vegetative growth response under salinity stress in the present meta-analysis was substantially higher in experiments in which multiple fungal species were used, rather than a single fungal species. It was previously shown that using different AM species in different wheat cultivars grown under salinity gave better results than using

individual AM species (Mardukhai *et al*., 2011). According to the last study, using a multiple AM species reduced chlorine and sodium uptake of the plant under salinity stress leading to enhanced plant growth (Mardukhai *et al*., 2011). However, this was not encountered with the mixed *G. mosseae* and *G. etunicatum* species. It is tempting to speculate that these two species might have been functionally redundant and did not complement each other in the root system, thus opposing the Functional Complementarity theory (Koide 2000). Alternatively, the ecological specificity can cause different fungal species to have different effects on plant growth or different susceptibility of the roots to colonisation by each fungus (McGonigle & Fitter 1990). Collectively, the findings presented here provide insights on how mycorrhizal effects on plants depend on specific environmental and fungal factors, and might explain the complexity of mycorrhizal function demonstrated in the previous chapters of this research investigation.

8.5 Conclusion

The meta-analysis of mycorrhizal experiments presented here provides substantial evidence and support gathered from various independent studies for the potential of arbuscular mycorrhizas to protect plants against salt stress, and remarkably increases our understanding of the diversity of mycorrhizal-plant interaction under salinity stress. The findings clearly revealed that AM inoculation was associated with a significant alleviation of the detrimental effects of salinity on plants. Across the examined studies, the complexity of the fungal inoculum (supplemented as single or multiple species) was found to be important in predicting plant responses to salt stress and generally suggested a superior impact for the multiple species inoculation in some situations. Interestingly, the overall results of the meta–analysis seem to align well with the theory of Functional Complementarity, which states that using multiple species of AM fungi will add more benefits to the plant (Koide 2000; Maherali & Klironomos 2007; Hoeksema *et al.*, 2010). Alternatively, mixed inocula may result in an increased likelihood for the beneficial fungi to exist in the mixed inoculum (Vogelsang *et al*., 2006; Hoeksema *et al.*, 2010). Similarly, Sharma *et al*. (1996) also confirmed the greater efficiency of using a collection of mycorrhizal species rather than a single species. This can be described as a ‘synergistic interaction’ between the species. Although the results in previous chapters

of this thesis were not conclusive regarding the potential advantages of multiple AM species (i.e. *G. mosseae* and *G. etunicatum*) over single species in combating salinity, the current meta-analysis clearly supports this notion. This is also in agreement with the results from a previously published meta–analysis (Hoeksema *et al*., 2010). Future research should exploit the ecological value of the positive interaction between mycorrhizas and plants under salinity conditions to improve the agricultural yield in saline soils, in addition to exploring the mechanistic basis of this symbiosis.

Chapter 9

General discussion

The present study aimed to investigate the effect of using mycorrhizal species to combat soil salinity and enhance plant growth under conditions of salinity stress. The effect of AM fungi on second generation plants and seed quality under salt stress was also explored. In addition to unravelling the significance of AM fungi in fighting soil salinity, which is critical for land reclamation, the long-term effects of mycorrhizas on plants in their first established generation and the offspring generation are particularly of interest and have been investigated in the present study. Moreover, it is important to understand the diversity of mycorrhizal species and their different behaviour with different plants under various stress conditions. Hence, single and multiple species of AM were included in the study to gain deeper insights into the species-specific responses of plants to AM fungi in the context of salinity stress.

Although the ecological and physiological aspects of arbuscular mycorrhizal fungal interactions with plants have been extensively researched, only a few studies have focused on the effect of AM fungi on the reproduction of a host plant. This includes flowers, seeds, and the survival of second generation plants in relation to the parental plant being colonised by AM fungi (Koide 2010). Preliminary experiments with the native wild plant *Sonchus oleraceus* (Chapter 3) showed that the association of *S. oleraceus* with mycorrhizas produced no enhancement of the growth of the parental plants and their offspring; yet, the morphological appearance of the second generation plants might point toward a beneficial impact of AM colonisation, possibly by favouring nutrient accumulation in *S. oleraceus* seeds and offspring, a suggestion that needs further investigation.

Because it is strongly mycorrhizal (mycotrophic), the native wild plant *Plantago lanceolata* was selected as a model plant. After intense work and several experiments, including a meta-analysis to investigate whether mycorrhizas can mitigate salt stress, the thesis found interesting points. In the initial experiments conducted under the controlled environment room conditions using NaCl or mixed salts to induce salinity stress (Chapter 4), the detrimental effects of salinity on plant establishment were

evident. Most plant growth parameters were significantly reduced by salinity, particularly at the highest salt level (10 dS/m). Furthermore, the negative impacts of mixed salts extended to offspring quality, causing a sharp decline in seed germination and seedling length. The most important factor determining the variability in the response of the plant to salt stress is that each salt type has a different effect on absorption of phosphorus from the soil (Gharineh *et al.*, 2009). The findings presented in Chapter 4 show the differential impact of salt type on seed yield, for example. Treatment with mixed salts in the second experiment caused a significant reduction in seed production compared with treatment with NaCl in the first experiment. Unlike mixed salts, which significantly altered the growth behaviour of the second generation, NaCl did not have any effect on offspring growth patterns. Another example is the first experiment of Chapter 5, in which using mixed salts reduced leaf number and shoot dry biomass to a greater extent than the NaCl treatment. In the same experiment, the production of seeds and inflorescence number were markedly enhanced by NaCl salt stress compared with treatment with mixed salts. Thus, it is crucial not to rely exclusively on NaCl as the main determinant of salinity stress, but rather mixed salt should be ideally used to mimic the real field situation.

Examining closely the interaction of mycorrhizas with *P. lanceolata* under salt stress in the present study highlighted the importance of salt composition, the microclimate, and fungal species type as critical factors determining the outcome of the mycorrhizal-plant relationship. Different mycorrhizas and plant species behave in a distinct manner with various types of salt (Hildebrandt *et al.*, 2001; Evelin *et al.*, 2009). Yet, most of the experiments examining the effect of salinity on mycorrhizas use NaCl as the main salinity inducer, which does not mimic the real situation where the plant is exposed to multiple ions and different compositions of salts in the soil (Zhu 2001). Interestingly, this study revealed notable differences in the results obtained by conducting mycorrhizal experiments under controlled environmental conditions, as in the CT room, and those obtained in a glasshouse and under field conditions. Most of the published mycorrhizal studies do not concentrate on the effect of small changes of microclimate on mycorrhizal associations with plants. The majority of the studies assume that AM fungi have similar physiological and ecological effects under field and laboratory conditions. Only few studies tried to show the differences in mycorrhizal behaviour under laboratory and field conditions (Buwalda *et al.*, 1982; Fitter 1985;

Yamato *et al*., 2008). Plant roots are known to behave differently under field and controlled conditions, especially with regard to the absorption of nitrogen (Gessler *et al.*, 1998). The experiments presented in Chapter 4 and Chapter 6, using commercial mycorrhizas under controlled room conditions, showed that the fungus spores failed to germinate and associate with the roots in the presence or absence of salinity stress. On the other hand, using similar strains of mycorrhizas in another experiment in a glasshouse resulted in successful colonisation of roots. Moreover, in the glasshouse, mycorrhizas reacted to salinity and managed to overcome stress for certain aspects of vegetative growth (Chapter 6). It was reported that a small rise in the soil temperature can alter mycorrhizal associations with the plant and increase carbon-nutrient exchange in the root system (Hawkes *et al.*, 2008). Consistent with this notion, the temperature in the glasshouse in our experiment was higher than the temperature in the CT room and the sunlight intensity was stronger than the laboratory lights, which might have promoted the association of roots with mycorrhizas, making colonisation more successful than in the experiments conducted under controlled conditions. However, as presented in Chapter 7, using two species of mycorrhizas (*Glomus etunicatum* and *G. mosseae*) gave successful colonisation under controlled conditions, unlike the previous experiments with commercial mixed mycorrhizas, thereby adding another level of complexity to the findings of the present investigation. Hence, for any future work with mycorrhizas, it is crucial to conduct the same experiment under different environmental conditions to ensure accuracy of the data, taking into consideration the variable responses of different species of mycorrhizas under the same environmental conditions.

The meta-analysis conducted in the present study (Chapter 8) consolidated evidence for a protective effect of AM against salinity and showed a superior role for the multiple inoculation in mitigating salt stress. However, using multiple species of mycorrhizas to fight salinity does not always result in positive enhancement of the plant compared with using a single AM species. The findings in previous chapters do not align well with the theory of Functional Complementarity (Koide 2000), as the results revealed that in some situations, using a single species of mycorrhiza could yield better results than the mixed inocula. Several studies indicate that different species of mycorrhizas have different functions during plant association (Munkvold *et al.*, 2004). In fact, the diversity of mycorrhizal species can trigger competition between the various species during root association and this, in turn, will not always be beneficial to the

plant. Another reason for the negative impact of using mixed mycorrhizas is that the plant root allocates only up to 20% of carbon to fungi in the symbiotic association (Schenk 2006). The addition of more species of mycorrhizas causes competition for the limited supply of carbon to the root, thereby depriving other plant growth organs of carbon (Buwalda & Koh 1982).

The mycorrhizas used in the present study only enhanced plant growth under mild salinity stress, while at high salinity the AM fungi failed to react. Previous studies have found that different species of mycorrhizas can tolerate only certain levels of salinity (Copeman *et al.*, 1996). Additionally, other experiments on lettuce and onion showed positive results for mycorrhizas up to medium salinity levels; beyond this level of salinity, the positive effect of the association began to disappear (Cantrell & Linderman 2001). Even though, there were few exceptions as it was found that mycorrhizal species can survive and thrive under severe salinity situations and react positively with plants to overcome the salt stress (Hilderbrandt *et al.*, 2000; Hoefnagels *et al.*, 1993). Collectively, the current results showed that the mycorrhizal species used in the experiments, which were obtained from a non-saline origin, performed better at low and medium levels of salinity stress. At high salinity levels, it would be better to use mycorrhizal species with a saline ecological background. Indeed, the use of mycorrhizas from saline habitats is known to be superior and was previously confirmed to give better results in enhancing plant resistance to salinity (Levy *et al*., 1983).

The species used in this set of experiments, *P. lanceolata*, is considered a glycophytic and not a halophytic plant (Schmitt *et al.*, 1992). This means that *P. lanceolata* is expected to be very sensitive to any changes in soil salinity and should react strongly. However, during the induction of salinity, the pattern of growth was not a linear decline but showed a bell-shaped curve instead. *P. lanceolata* exhibited relatively improved growth in low salinity (1–3 dS/m), but a further increase of salinity negatively influenced plant growth, regardless of the addition of mycorrhizas. Halophytic plant species are capable of tolerating salinity even at high levels because these plants can adapt physiologically and genetically to salt stress and react to it positively (Glen *et al.*, 1999). Yet, there are a few exceptions, as some non-saline plants can react positively to certain levels of salinity stress (Duke *et al.*, 1986). It is believed that at low levels of salinity, some of the ions such as sodium can act as a fertiliser, thus providing nutritional value for plants and enhancing their growth (Mengel & Kirkby

1982). Therefore, even without the addition of mycorrhizas to overcome the salinity stress at low and medium levels of salinity, *P. lanceolata* exhibited a positive growth pattern in some experiments.

A limited number of studies exploring the effect of mycorrhizas on seeds and second generation plants are currently available, probably due to the long time frame needed to conduct experiments over successive generations. The results of the effect of mycorrhizas and salinity stress on plant offspring were complex and not conclusive. Seed germination was enhanced by commercial mixed AM, whereas the combination of *G. etunicatum* and *G. mosseae* had little overall impact on second generation seedlings, as demonstrated in the field experiments (Chapter 5). As presented in Chapter 7, using a single species of mycorrhiza did lead to some changes in the second generation, particularly the addition of *G. mosseae* under no salt stress. As detailed in the same chapter, *G. mosseae* inoculation of parental plants resulted in less seedling growth and less vigorous seed germination under salinity treatments. The results from several experiments suggest that there are many other factors, such as salt type and salinity levels, which are more important for determining offspring behaviour than the addition of AM fungi alone.

Mycorrhizal colonisation was reduced with increasing salinity stress, which is consistent with previous reports (McMillen *et al*., 1998; Juniper & Abbott, 1993; Kaya *et al.*, 2009). It was expected that under salinity stress, mycorrhizal spores would fail to germinate or have low potential for germination (Juniper & Abbott 2006). Also, the same study shows that high levels of salinity can cause ion toxicity and reduce the osmotic potential of the root cells, which disrupt the association with mycorrhizas. Moreover, in some situations with increasing salinity stress, the root produces less carbohydrate as a source of carbon for the stimulation of mycorrhizal growth, and hence the rate of root colonisation decreases (Amijee *et al.*, 1993; Wright *et al.*, 1998b). Thus, the data obtained in this thesis confirm that higher levels of salinity stress reduce mycorrhizal functionality and decrease the formation of symbiotic associations with the plant. On the other hand, as mycorrhizal colonisation decreases with increasing salinity levels, AM fungal spore production increases. As described in Chapter 5, the first field experiment showed increased numbers of spores under medium and high salinity levels, in particular. It is known that under stressful situations, mycorrhizas tend to produce more spores (Guadarrama & Alvarez-Sanchez 1999; Ortega-Larrocea *et al.*, 2001).

Accordingly, it is tempting to speculate that under high salinity stress, instead of forming a successful symbiotic association with the root, the mycorrhizal inoculum used in this experiment diverted its resources into spore production.

In conclusion, despite the complexity of the mycorrhizal-plant interaction in these experiments, the work presented in this thesis provides tantalising insights into the role of mycorrhizas in alleviating salt stress under different experimental settings. The findings improve our understanding of the responses of first and second generation plants to mycorrhizas in the context of salinity stress and under various environmental conditions, and shed light on the importance of several factors, such as salt type, microclimate, and fungal inoculum complexity, in determining the outcome of the fungus-plant relationship under salt stress, thus opening new horizons for future research.

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Appendix A

Miracle – Gro concentrated liquid plant food solution contains:

NPK Fertiliser solution 6-5-5

Total Nitrogen (N) 6%.

Nitric nitrogen (N) 3.2%

Ammoniacal nitrogen (N) 2.8%.

Phosphorus pentoxide (P_2O_5) soluble in water 5% (2.2 % P)

Potassium oxide (K2O) soluble in water 5% (4.1%K)

Copper (Cu) soluble in water, chelated by EDTA 0.002%

Iron (Fe) soluble in water chelated by DTPA 0.03%

Manganese (Mn) soluble in water, chelated by EDTA 0.01%

Molybdenum (Mo) soluble in water 0.001%

Zinc (Zn) soluble in water, chelated by EDTA 0.002%

Appendix B

Preparation of salinity solutions

1) Sodium Chloride (NaCl) solutions.

- Our salinity solutions Electrical conductivity values:
  - 2.2 dS/m (light salinity solution)
  - 5 dS/m (medium salinity solution)
  - 10 dS/m (high saline solution)

A) 2.2 dS/m

Gram= L x Molarity mol/L x molecular formula (NaCl) g/mol

g= 8 L x 0.0202 mol/L x 58.44 g/mol

= 9.44 g

B) 5 dS/m

Gram= 8 L x 0.05 mol/L x 58.44 g/mol

= 23.4 g

C) 10 dS/m

Gram = 8 L x 0.1 mol/L x 58.44 g/mol

= 46.8 g

2) Mixed sea salt solution

- Our salinity solution Electrical conductivity values:
    - 1 dS/m
    - 1.5 dS/m
    - 1.7 dS/m
    - 2.2 dS/m
    - 3.5 dS/m
    - 5 dS/m (medium salinity solution)
    - 10 dS/m (high saline solution)

- The molecular formula of sea salt is not available, but we have stock solution 46.5 dS/m.

A) 1 dS/m.

$m_1 v_1 = M_2 V_2$

46.5 dS/m x v_1 = 1 dS/m x 3 L

V_1 = 0.6 L (stock solution)

3 L – 0.06 L = 2.94 L (distilled water)

B) 1.5 dS/m.

46.5 dS/m x V_1 = 1.5 dS/m x 7.200 L

V_1 = 0.232 L (stock solution)

7.200 L – 0.232 L = 6.968 L (distilled water)

C) 1.7 dS/m.

46.5 dS/m x V_1 = 1.7 dS/m x 3 L

V_1 = 0.110 L (stock solution)

3 L – 0.110 L = 2.89 L (distilled water)

D) 2.2 dS/m.

46.5 dS/m x V_1 = 2.2 dS/m x 3L

V_1 = 0.142 L (stock solution)

3L – 0.142 L = 2.860 L (distilled water)

E) 3.5 dS/m

46.5 dS/m x V_1 = 3.5 dS/m x 7.200 L

V_1 = 0.542 L (stock solution)

7.200 L – 0.542 L = 6.658 L (distilled water)

F) 5 dS/m.

46.5 dS/m x V_1 = 5 dS/m x 3 L

V_1 = 0.322 L (Stock solution)

3L – 0.322 L = 2.680 L (distilled water)

G) 10 dS/m.

46.5 dS/m x V_1 = 10 dS/m x 3 L

V_1 = 0.645 L (stock solution)

3L – 0.645 L = 2.360 L (distilled solution)

Appendix C

The Standardised Mean Difference (Hedges's *d*) equation:

$$\overline{E} = \frac{\overline{X}_{G1} - \overline{X}_{G2}}{s_{pooled}} \quad J(m)$$

$$s_{pooled} = \sqrt{\frac{s_1^2(n_1 - 1) + s_2^2(n_2 - 1)}{n_1 + n_2 - 2}}$$

$\overline{E}$ is the effect size or the standardised mean difference, *d*; $\overline{X}_{G1}$ and $\overline{X}_{G2}$ are the means of each group; n_1 and n_2 are groups sample sizes; S_1 and S_2 are standard deviations in each group; S_{pooled} is the pooled standard deviation of the groups; *J(m)* is a correction factor for small sample bias (Hedges & Olkin 1985).

After calculating the standardised mean difference (or effect size) between the treatment and control group in each study by Hedges's *d* equation, the calculated effect sizes are summarised in the meta-analysis by calculating the mean of these data points. However, differences in sample sizes and precision (standard error) across different experiments necessitate the use of a weighted mean because typically studies with larger samples sizes will have lower variances, and thus will provide more precise estimates of the true population effect size (e.g., Hedges 1983). Therefore, a weighted average is used in meta-analysis to estimate the combined effect size for a sample of studies, where the weight for the i^{th} study is the reciprocal of its sampling variance, $\omega_i = 1/v_i$, where *vi* is the within-study variance for study (*i*). The combined effect size (weighted mean) is then computed as:

$$\overline{E} = \frac{\sum_{i=1}^{n} \omega i \; Ei}{\sum_{i=1}^{n} \omega i}$$

that is, the sum of the products *ωiEi* (effect size multiplied by weight) divided by the sum of the weights, where *n* is the number of studies and *Ei* is the effect size for the i^{th} study.

The variance of the combined effect is defined as the reciprocal of the sum of the weights, or:

$$v = \frac{1}{\sum_{i=1}^{n} \omega i}$$

and the standard error of the combined effect is then the square root of the variance:

$$SE(\bar{E}) = \sqrt{v}$$

The 95% confidence interval for the combined effect would be computed as:

$$\textbf{Lower Limit} = \bar{E} - 1.96 * SE(\bar{E})$$
$$\textbf{Upper Limit} = \bar{E} + 1.96 * SE(\bar{E})$$

Finally, if desired, the *Z*-value could be computed using:

$$Z = \frac{\bar{E}}{SE(\bar{E})}$$

For a two-tailed test the p-value would be given by:

$$p = 2\,[1 - (\Phi(|\,Z\,|))]$$

Where Φ is the standard normal cumulative distribution function.

With this confidence interval, the overall effect present in a set of studies can now be evaluated. The cumulative effect size represents the overall magnitude of the effect present in the studies; this value is considered to be significantly different from zero if its confidence limits do not bracket zero (i.e., $\bar{E}$ is significant at $p<0.05$). Thus, with the combined mean effect size and its confidence interval, we can determine whether there is significant evidence supporting a particular hypothesis, and also estimate the magnitude of that support (Rosenberg *et al.*, 2000).